

**ANTISENSE OLIGONUCLEOTIDE MODULATION OF
TUMOR NECROSIS FACTOR- α (TNF- α) EXPRESSION**

5 INTRODUCTION

This application is a continuation-in-part of U.S. Application Serial No. 09/824,322, filed April 2, 2001, which is a continuation-in part of allowed U.S. Application Serial No. 09/313,932, filed May 18, 1999 (U.S. Patent 6,228,642),
10 which is a continuation-in-part of U. S. Application Serial No. 09/166,186 filed October 5, 1998 (U.S. Patent No. 6,080,580).

FIELD OF THE INVENTION

15 This invention relates to compositions and methods for modulating expression of the human tumor necrosis factor- α (TNF- α) gene, which encodes a naturally present cytokine involved in regulation of immune function and implicated in infectious and inflammatory disease. This invention is also
20 directed to methods for inhibiting TNF- α mediated immune responses; these methods can be used diagnostically or therapeutically. Furthermore, this invention is directed to treatment of conditions associated with expression of the human TNF- α gene.

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BACKGROUND OF THE INVENTION

Tumor necrosis factor α (TNF- α also cachectin) is an important cytokine that plays a role in host defense. The cytokine is produced primarily in macrophages and monocytes in
30 response to infection, invasion, injury, or inflammation. Some examples of inducers of TNF- α include bacterial endotoxins, bacteria, viruses, lipopolysaccharide (LPS) and cytokines including GM-CSF, IL-1, IL-2 and IFN- γ .

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TNF- α interacts with two different receptors, TNF receptor I (TNFRI, p55) and TNFRII (p75), in order to transduce its effects, the net result of which is altered gene expression. Cellular factors induced by TNF- α include

5 interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), interferon- γ (IFN- γ), platelet derived growth factor (PDGF) and epidermal growth factor (EGF), and endothelial cell adhesion molecules including endothelial leukocyte adhesion molecule 1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-
10 1) and vascular cell adhesion molecule-1 (VCAM-1) (Tracey, K.J., et al., Annu. Rev. Cell Biol., **1993**, 9, 317-343; Arvin, B., et al., Ann. NY Acad. Sci., **1995**, 765, 62-71).

Despite the protective effects of the cytokine, overexpression of TNF- α often results in disease states,
15 particularly in infectious, inflammatory and autoimmune diseases. This process may involve the apoptotic pathways (Ksontini, R., et al., J. Immunol., **1998**, 160, 4082-4089). High levels of plasma TNF- α have been found in infectious diseases such as sepsis syndrome, bacterial meningitis,
20 cerebral malaria, and AIDS; autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease (including Crohn's disease), sarcoidosis, multiple sclerosis, Kawasaki syndrome, graft-versus-host disease and transplant (allograft) rejection; and organ failure conditions such as adult
25 respiratory distress syndrome, congestive heart failure, acute liver failure and myocardial infarction (Eigler, A., et al., Immunol. Today, **1997**, 18, 487-492). Other diseases in which TNF- α is involved include asthma (Shah, A., et al., Clinical and Experimental Allergy, **1995**, 25, 1038-1044), brain injury
30 following ischemia (Arvin, B., et al., Ann. NY Acad. Sci., **1995**, 765, 62-71), non-insulin-dependent diabetes mellitus (Hotamisligil et al., Science, **1993**, 259, 87-90), insulin-dependent diabetes mellitus (Yang et al., J. Exp. Med., **1994**, 180, 995-1004), hepatitis (Ksontini et al., J. Immunol., **1998**,

160, 4082-4089), atopic dermatitis (Sumimoto et al., Arch. Dis. Child., 1992, 67, 277-279), and pancreatitis (Norman et al., Surgery, 1996, 120, 515-521). Further, inhibitors of TNF- α have been suggested to be useful for cancer prevention
5 (Suganuma et al. (*Cancer Res.*, 1996, 56, 3711-3715). Elevated TNF- α expression may also play a role in obesity (Kern, J. Nutr., 1997, 127, 1917S-1922S). TNF- α was found to be expressed in human adipocytes and increased expression, in general, correlated with obesity.

10 There are currently several approaches to inhibiting TNF- α expression. Approaches used to treat rheumatoid arthritis include a chimeric anti-TNF- α antibody, a humanized monoclonal anti-TNF- α antibody, and recombinant human soluble TNF- α receptor (Camussi, *Drugs*, 1998, 55, 613-620). Other
15 examples are indirect TNF- α inhibitors including phosphodiesterase inhibitors (e.g., pentoxifylline) and metalloprotease inhibitors (Eigler et al., *Immunol. Today*, 1997, 18, 487-492). An additional class of direct TNF- α inhibitors is oligonucleotides, including triplex-forming
20 oligonucleotides, ribozymes, and antisense oligonucleotides. Several publications describe the use of oligonucleotides targeting TNF- α by non-antisense mechanisms. U.S. Patent 5,650,316, WO 95/33493 and Aggarwal et al. (*Cancer Research*, 1996, 56, 5156-5164) disclose triplex-forming oligonucleotides
25 targeting TNF- α . WO 95/32628 discloses triplex-forming oligonucleotides especially those possessing one or more stretches of guanosine residues capable of forming secondary structure. WO 94/10301 discloses ribozyme compounds active against TNF- α mRNA. WO 95/23225 discloses enzymatic nucleic
30 acid molecules active against TNF- α mRNA.

A number of publications have described the use of antisense oligonucleotides targeting nucleic acids encoding TNF- α . The TNF- α gene has four exons and three introns. WO

93/09813 discloses TNF- α antisense oligonucleotides conjugated to a radioactive moiety, including sequences targeted to the 5'-UTR, AUG start site, exon 1, and exon 4 including the stop codon of human TNF- α . EP 0 414 607 B1 discloses antisense oligonucleotides targeting the AUG start codon of human TNF- α . WO 95/00103 claims antisense oligonucleotides to human TNF- α including sequences targeted to exon 1 including the AUG start site. Hartmann et al. (*Mol. Med.*, **1996**, 2, 429-438) disclose uniform phosphorothioates and mixed backbone phosphorothioate/phosphodiester oligonucleotides targeted to the AUG start site of human TNF- α . Hartmann et al. (*Antisense Nucleic Acid Drug Devel.*, **1996**, 6, 291-299) disclose antisense phosphorothioate oligonucleotides targeted to the AUG start site, the exon 1/intron 1 junction, and exon 4 of human TNF- α . d'Hellencourt et al. (*Biochim. Biophys. Acta*, **1996**, 1317, 168-174) designed and tested a series of unmodified oligonucleotides targeted to the 5'-UTR, and exon 1, including the AUG start site, of human TNF- α . Additionally, one oligonucleotide each was targeted to exon 4 and the 3'-UTR of human TNF- α and one oligonucleotide was targeted to the AUG start site of mouse TNF- α . Rojanasakul et al. (*J. Biol. Chem.*, **1997**, 272, 3910-3914) disclose an antisense phosphorothioate oligonucleotide targeted to the AUG start site of mouse TNF- α . Taylor et al. (*J. Biol. Chem.*, **1996**, 271, 17445-17452 and *Antisense Nucleic Acid Drug Devel.*, **1998**, 8, 199-205) disclose morpholino, methyl-morpholino, phosphodiester and phosphorothioate oligonucleotides targeted to the 5'-UTR and AUG start codon of mouse TNF- α . Tu et al. (*J. Biol. Chem.*, **1998**, 273, 25125-25131) designed and tested 42 phosphorothioate oligonucleotides targeting sequences throughout the rat TNF- α gene.

Interestingly, some phosphorothioate oligodeoxynucleotides have been found to enhance

lipopolysaccharide-stimulated TNF- α synthesis up to four fold due to nonspecific immunostimulatory effects (Hartmann et al. *Mol. Med.*, 1996, 2, 429-438).

Accordingly, there remains an unmet need for therapeutic
5 compositions and methods for inhibiting expression of TNF- α , and disease processes associated therewith.

SUMMARY OF THE INVENTION

The present invention provides oligonucleotides which
10 are targeted to nucleic acids encoding TNF- α and are capable of modulating TNF- α expression. The present invention also provides chimeric oligonucleotides targeted to nucleic acids encoding human TNF- α . The oligonucleotides of the invention are believed to be useful both diagnostically and
15 therapeutically, and are believed to be particularly useful in the methods of the present invention.

The present invention also comprises methods of modulating the expression of human TNF- α in cells and tissues using the oligonucleotides of the invention. Methods of
20 inhibiting TNF- α expression are provided; these methods are believed to be useful both therapeutically and diagnostically. These methods are also useful as tools, for example, for detecting and determining the role of TNF- α in various cell functions and physiological processes and conditions and for
25 diagnosing conditions associated with expression of TNF- α .

The present invention also comprises methods for diagnosing and treating infectious and inflammatory diseases, particularly diabetes, rheumatoid arthritis, Crohn's disease, pancreatitis, multiple sclerosis, atopic dermatitis and
30 hepatitis using the oligonucleotides of the present invention. These methods are believed to be useful, for example, in diagnosing TNF- α -associated disease progression. These methods are believed to be useful both therapeutically,

including prophylactically, and as clinical research and diagnostic tools.

One embodiment of the present invention is a method of treating an inflammatory disorder in an individual comprising administering to said individual an effective amount of an oligonucleotide up to 30 nucleotides in length complementary to a nucleic acid molecule encoding human tumor necrosis factor- α , wherein the oligonucleotide inhibits the expression of said human tumor necrosis factor- α and comprises at least an 8 nucleobase portion of SEQ ID NO: 24, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 34, SEQ ID NO: 39, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 149, SEQ ID NO: 157, SEQ ID NO: 264, SEQ ID NO: 271, SEQ ID NO: 272, SEQ ID NO: 290, SEQ ID NO: 297, SEQ ID NO: 299, SEQ ID NO: 315, SEQ ID NO: 334, SEQ ID NO: 418, SEQ ID NO: 423, SEQ ID NO: 425, SEQ ID NO: 427, SEQ ID NO: 431, SEQ ID NO: 432, SEQ ID NO: 435, SEQ ID NO: 437, SEQ ID NO: 438, SEQ ID NO: 439, SEQ ID NO: 441, SEQ ID NO: 455, SEQ ID NO: 457, SEQ ID NO: 458, SEQ ID NO: 460, SEQ ID NO: 463, SEQ ID NO: 465, SEQ ID NO: 466, SEQ ID NO: 468, SEQ ID NO: 472, SEQ ID NO: 474, SEQ ID NO: 475, SEQ ID NO: 483, SEQ ID NO: 485, SEQ ID NO: 494 or SEQ ID NO: 496. Preferably, the antisense oligonucleotide is administered orally. In one aspect of this preferred embodiment, the inflammatory disorder is inflammatory bowel disease, Crohn's disease, colitis or rheumatoid arthritis. Preferably, the oligonucleotide comprises at least one modified intersugar linkage. Preferably, the modified intersugar linkage is a phosphorothioate or methylene(methylimino) intersugar linkage. In another aspect of this preferred embodiment, the oligonucleotide comprises at least one 2'-O-methoxyethyl modification. Preferably, the oligonucleotide comprises at least one 5-methyl cytidine. In one aspect of this preferred embodiment, every cytidine residue is a 5-methyl cytidine.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-B are graphs showing collagen-induced arthritis (CIA) onset as determined by percent incidence in mice. Incidence=number of mice with at least one affected paw/total number of mice per group. Figure 1A shows the effect of low dose range of ISIS 25302 anti-TNF- α antisense oligonucleotide in comparison to treatment by an anti-TNF- α mAb. Figure 1B shows the effect of high dose range treatment by ISIS 25302 in comparison to treatment by an 8 mismatch control oligonucleotide (ISIS 30782).

Figure 2 is a graph showing "total" histological scores for colon tissue from IL-10^{-/-} mice treated with saline (vehicle), ISIS 25302 or 8MM Con. As recorded in Table 27. Results are expressed as mean " standard deviation (n=6). The asterisk indicates a significant difference (p < 0.05) in comparison to the vehicle group.

Figures 3A-B show the basal (Fig. 3A) and LPS-induced (Fig. 3B) levels of TNF- α secretion from colon tissue of IL-10^{-/-} mice post-treatment with ISIS 25302 and the 8 base mismatch control oligonucleotide 30782 (8MM). Doses of oligonucleotide are shown in parentheses (mg/kg). Secretion levels (pg/gm-tissue) are shown in the y-axis. The mean values " standard deviation (n=7 to 9) are shown.

Figures 4A-B show the basal (Fig. 4A) and LPS-induced (Fig. 4B) levels of IFN- γ secretion from colon tissue of IL-10^{-/-} mice post-treatment with ISIS 25302 and the 8 base mismatch control oligonucleotide 30782 (8MM). Doses of oligonucleotide are shown in parentheses (mg/kg). Secretion levels (pg/gm-tissue) are shown in the y-axis. The mean values " standard deviation (n=6 to 9) are shown.

Figures 5A-B show the efficacy of ISIS 25302 versus anti-mouse TNF- α mAb in the acute model of DSS-induced colitis. Fig. 5A shows the disease activity index (DAI). Fig. 5B shows the effect of different treatments on colon

length. Results are expressed as the mean " S.E.M., where n=7. Asterisks show a significant difference from saline treated (*) or normal (*) group ($p<0.05$).

Figures 6A-B show that the prevention of acute colitis by ISIS 25302 in the DSS-induced colitis molecule is sequence-dependent. Fig. 5A shows DAI versus treatment. Fig. 5B shows the effect of different treatments on colon length. Asterisks indicate significant differences from saline (*) or 1.0 mg/kg 8MM Con (*) treated group ($p<0.05$).

Figures 6A-B are graphs showing the efficacy of ISIS 25302 in the DSS-induced mouse model of chronic colitis based on DAI. Fig. 6A shows the mean DAI of each group over the course of the two cycle DSS-induced chronic colitis study. Fig. 6B shows the mean DAI at representative cycle times. The doses are indicated in parentheses (mg/kg). Results are expressed as the mean S.E.M., where $n=8$ to 10 . Asterisks indicate statistical significance in comparison to the Vehicle group ($P<0.05$).

Figures 8A-B show histopathology of colon tissue from mice administered DSS in the two cycle chronic colitis model. Results are expressed as mean S.E.M. Fig. 8A shows the total inflammation and crypt scores. Acute inflammatory infiltrates consist of granulocytes, lymphocytes and plasma cells. Chronic inflammatory infiltrates consist of granulocytes, lymphocytes, plasma cells, monocytes and macrophages. Fig. 8B shows histological scores of different regions of the colon. PA=proximal acute inflammation score, DA=distal acute inflammation score, PC= proximal chronic inflammation score, DC=distal chronic inflammation score, PCS=proximal crypt score and DCS=distal crypt score. Asterisks indicate statistical significance in comparison to the Vehicle group ($p<0.05$).

Figure 9 shows TNF- α mRNA levels from longitudinal sections of colon tissue derived from each mouse at time of sacrifice in the chronic colitis model (mean S.E.M.). Group A=0.25 mg/kg ISIS 25302, group B=Vehicle, group C=anti-TNF

mAb, group D=no treatment, group E=2.5 mg/kg ISIS 25302, group F=12.5 mg/kg ISIS 25302.

DETAILED DESCRIPTION OF THE INVENTION

5 TNF- α plays an important regulatory role in the immune response to various foreign agents. Overexpression of TNF- α results in a number of infectious and inflammatory diseases. As such, this cytokine represents an attractive target for treatment of such diseases. In particular, modulation of the
10 expression of TNF- α may be useful for the treatment of diseases such as Crohn's disease, diabetes mellitus, multiple sclerosis, rheumatoid arthritis, hepatitis, pancreatitis and asthma.

 The present invention employs antisense compounds,
15 particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding TNF- α , ultimately modulating the amount of TNF- α produced. This is accomplished by providing oligonucleotides which specifically hybridize with nucleic acids, preferably mRNA, encoding TNF- α .

20 This relationship between an antisense compound such as an oligonucleotide and its complementary nucleic acid target, to which it hybridizes, is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid target, in the context of this invention, is a
25 multistep process. The process usually begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease state, or a foreign nucleic acid from an
30 infectious agent. In the present invention, the targets are nucleic acids encoding TNF- α ; in other words, a gene encoding TNF- α , or mRNA expressed from the TNF- α gene. mRNA which encodes TNF- α is presently the preferred target. The targeting process also includes determination of a site or sites within

the nucleic acid sequence for the antisense interaction to occur such that modulation of gene expression will result.

In accordance with this invention, persons of ordinary skill in the art will understand that messenger RNA includes not only the information to encode a protein using the three letter genetic code, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region and intron/exon junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the informational ribonucleotides. The oligonucleotide may therefore be specifically hybridizable with a transcription initiation site region, a translation initiation codon region, a 5' cap region, an intron/exon junction, coding sequences, a translation termination codon region or sequences in the 5'- or 3'-untranslated region. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or

codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding TNF- α , regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region," "AUG region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. This region is a preferred target region. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. This region is a preferred target region. The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other preferred target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap

structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly
5 translated, many contain one or more regions, known as
"introns," which are excised from a pre-mRNA transcript to
yield one or more mature mRNAs. The remaining (and therefore
translated) regions are known as "exons" and are spliced
together to form a continuous mRNA sequence. mRNA splice
10 sites, i.e., exon-exon or intron-exon junctions, may also be
preferred target regions, and are particularly useful in
situations where aberrant splicing is implicated in disease,
or where an overproduction of a particular mRNA splice product
is implicated in disease. Aberrant fusion junctions due to
15 rearrangements or deletions are also preferred targets.
Targeting particular exons in alternatively spliced mRNAs may
also be preferred. It has also been found that introns can
also be effective, and therefore preferred, target regions for
antisense compounds targeted, for example, to DNA or pre-mRNA.

20 Once the target site or sites have been identified,
oligonucleotides are chosen which are sufficiently
complementary to the target, i.e., hybridize sufficiently well
and with sufficient specificity, to give the desired
modulation.

25 "Hybridization", in the context of this invention, means
hydrogen bonding, also known as Watson-Crick base pairing,
between complementary bases, usually on opposite nucleic acid
strands or two regions of a nucleic acid strand. Guanine and
cytosine are examples of complementary bases which are known
30 to form three hydrogen bonds between them. Adenine and
thymine are examples of complementary bases which form two
hydrogen bonds between them.

"Specifically hybridizable" and "complementary" are
terms which are used to indicate a sufficient degree of

complementarily such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide.

It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarily to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment or, in the case of *in vitro* assays, under conditions in which the assays are conducted.

Hybridization of antisense oligonucleotides with mRNA interferes with one or more of the normal functions of mRNA. The functions of mRNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in by the RNA. Binding of specific protein(s) to the RNA may also be interfered with by antisense oligonucleotide hybridization to the RNA.

The overall effect of interference with mRNA function is modulation of expression of TNF- α . In the context of this invention "modulation" means either inhibition or stimulation; i.e., either a decrease or increase in expression. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression, or reverse transcriptase PER, as taught in the examples of the instant application or by Western blot or ELIZA assay of protein expression, or by an immunoprecipitation assay of protein expression. Effects of antisense oligonucleotides of the present invention on TNF- α expression can also be

determined as taught in the examples of the instant application. Inhibition is presently a preferred form of modulation.

While the preferred form of antisense compound is a
5 single-stranded antisense oligonucleotide, in many species the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This
10 phenomenon occurs in both plants and animals and is believed to have an evolutionary connection to viral defense and transposon silencing.

The first evidence that dsRNA could lead to gene silencing in animals came in 1995 from work in the nematode,
15 *Caenorhabditis elegans* (Guo and Kempheus, *Cell*, **1995**, 81, 611-620). Montgomery et al. have shown that the primary interference effects of dsRNA are posttranscriptional (Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **1998**, 95, 15502-15507). The posttranscriptional antisense mechanism
20 defined in *Caenorhabditis elegans* resulting from exposure to double-stranded RNA (dsRNA) has since been designated RNA interference (RNAi). This term has been generalized to mean antisense-mediated gene silencing involving the introduction of dsRNA leading to the sequence-specific reduction of
25 endogenous targeted mRNA levels (Fire et al., *Nature*, **1998**, 391, 806-811). Recently, it has been shown that it is, in fact, the single-stranded RNA oligomers of antisense polarity of the dsRNAs which are the potent inducers of RNAi (Tijsterman et al., *Science*, **2002**, 295, 694-697). The use of
30 these dsRNAs targeted to nucleic acid encoding TNF- α is also within the scope of the present invention. These dsRNAs target the same or similar regions to those targeted by antisense oligonucleotides.

The oligonucleotides of the present invention also
35 include variants in which a different base is present at one

or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenosine, variants may be produced which contain thymidine, guanosine or cytidine at this position. This may be done at any of the positions of the oligonucleotide. These oligonucleotides are then tested using the methods described herein to determine their ability to inhibit expression of TNF- α .

The oligonucleotides of this invention can be used in diagnostics, therapeutics, prophylaxis, and as research reagents and in kits. Since the oligonucleotides of this invention hybridize to nucleic acids encoding TNF- α , sandwich, colorimetric and other assays can easily be constructed to exploit this fact. Provision of means for detecting hybridization of oligonucleotides with the TNF- α gene or mRNA can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of TNF- α may also be prepared.

The present invention is also suitable for diagnosing abnormal inflammatory states in tissue or other samples from patients suspected of having an inflammatory disease such as rheumatoid arthritis. The ability of the oligonucleotides of the present invention to inhibit inflammatory processes may be employed to diagnose such states. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection and, usually, quantitation of such inhibition. In the context of this invention, to "contact" tissues or cells with an oligonucleotide or oligonucleotides means to add the oligonucleotide(s), usually in a liquid carrier, to a cell suspension or tissue sample, either *in vitro* or *ex vivo*, or to administer the oligonucleotide(s) to cells or tissues within an animal.

The oligonucleotides of this invention may also be used for research purposes. Thus, the specific hybridization exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations and in other
5 methodologies which may be appreciated by persons of ordinary skill in the art.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes
10 oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because
15 of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

The antisense compounds in accordance with this invention preferably comprise from about 5 to about 50
20 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e., from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is
25 normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidine. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a
30 pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear
35 polymeric structure can be further joined to form a circular

structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal
5 linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages.
10 As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides
15 that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Oligomer and Monomer Modifications

As is known in the art, a nucleoside is a base-sugar
20 combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the
25 nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric
30 compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may have internal nucleobase complementarity and may therefore fold in a manner as to
35 produce a fully or partially double-stranded compound. Within

oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside linkage or in conjunction with the sugar ring the backbone of the oligonucleotide. The normal internucleoside linkage that makes up the backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Modified Internucleoside Linkages

Specific examples of preferred antisense oligomeric compounds useful in this invention include oligonucleotides containing modified e.g. non-naturally occurring internucleoside linkages. As defined in this specification, oligonucleotides having modified internucleoside linkages include internucleoside linkages that retain a phosphorus atom and internucleoside linkages that do not have a phosphorus atom. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

In the *C. elegans* system, modification of the internucleotide linkage (phosphorothioate) did not significantly interfere with RNAi activity. Based on this observation, it is suggested that certain preferred oligomeric compounds of the invention can also have one or more modified internucleoside linkages. A preferred phosphorus containing modified internucleoside linkage is the phosphorothioate internucleoside linkage.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates,

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thionoalkylphosphotriesters, selenophosphates and borano-phosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In more preferred embodiments of the invention, oligomeric compounds have one or more phosphorothioate and/or heteroatom internucleoside linkages, in particular -CH₂-NH-O-CH₂-, -CH₂-N(CH₃)-O-CH₂- [known as a methylene (methylimino) or MMI backbone], -CH₂-O-N(CH₃)-CH₂-, -CH₂-N(CH₃)-CH₂- and -O-N(CH₃)-CH₂-CH₂- [wherein the native phosphodiester internucleotide linkage is represented as -O-P(=O)(OH)-O-CH₂-]. The MMI type internucleoside linkages are disclosed in the above referenced U.S. patent 5,489,677. Preferred amide internucleoside linkages are disclosed in the above referenced U.S. patent 5,602,240.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside

linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

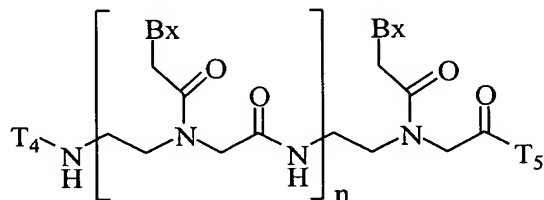
Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Oligomer Mimetics

Another preferred group of oligomeric compounds amenable to the present invention includes oligonucleotide mimetics. The term mimetic as it is applied to oligonucleotides is intended to include oligomeric compounds wherein only the furanose ring or both the furanose ring and the internucleotide linkage are replaced with novel groups, replacement of only the furanose ring is also referred to in the art as being a sugar surrogate. The heterocyclic base moiety or a modified heterocyclic base moiety is maintained for hybridization with an appropriate target nucleic acid. One such oligomeric compound, an oligonucleotide mimetic that

has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA oligomeric compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA oligomeric compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA oligomeric compounds can be found in Nielsen *et al.*, *Science*, **1991**, 254, 1497-1500.

PNA has been modified to incorporate numerous modifications since the basic PNA structure was first prepared. The basic structure is shown below:



wherein

Bx is a heterocyclic base moiety;

T₄ is hydrogen, an amino protecting group, -C(O)R₅, substituted or unsubstituted C₁-C₁₀ alkyl, substituted or unsubstituted C₂-C₁₀ alkenyl, substituted or unsubstituted C₂-C₁₀ alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group, a reporter group, a conjugate group, a D or L α-amino acid linked via the α-carboxyl group or optionally through the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

T_5 is $-OH$, $-N(Z_1)Z_2$, R_5 , D or L α -amino acid linked via the α -amino group or optionally through the ω -amino group when the amino acid is lysine or ornithine or a peptide derived from D, L or mixed D and L amino acids linked through an amino group, a chemical functional group, a reporter group or a conjugate group;

Z_1 is hydrogen, C_1-C_6 alkyl, or an amino protecting group;

Z_2 is hydrogen, C_1-C_6 alkyl, an amino protecting group, $-C(=O)-(CH_2)_n-J-Z_3$, a D or L α -amino acid linked via the α -carboxyl group or optionally through the ω -carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group;

Z_3 is hydrogen, an amino protecting group, $-C_1-C_6$ alkyl, $-C(=O)-CH_3$, benzyl, benzoyl, or $-(CH_2)_n-N(H)Z_1$;

each J is O, S or NH;

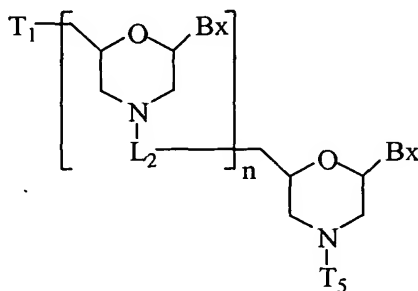
R_5 is a carbonyl protecting group; and

n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been studied is based on linked morpholino units (morpholino nucleic acid) having heterocyclic bases attached to the morpholino ring. A number of linking groups have been reported that link the morpholino monomeric units in a morpholino nucleic acid. A preferred class of linking groups have been selected to give a non-ionic oligomeric compound. The non-ionic morpholino-based oligomeric compounds are less likely to have undesired interactions with cellular proteins. Morpholino-based oligomeric compounds are non-ionic mimics of oligonucleotides which are less likely to form undesired interactions with cellular proteins (Dwaine A. Braasch and David R. Corey, *Biochemistry*, **2002**, *41*(14), 4503-4510). Morpholino-based oligomeric compounds are disclosed in United States Patent 5,034,506, issued July 23, 1991. The morpholino class of oligomeric compounds have been prepared having a

variety of different linking groups joining the monomeric subunits.

Morpholino nucleic acids have been prepared having a variety of different linking groups (L_2) joining the monomeric subunits. The basic formula is shown below:



wherein

T_1 is hydroxyl or a protected hydroxyl;

T_5 is hydrogen or a phosphate or phosphate derivative;

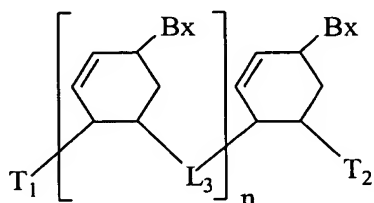
L_2 is a linking group; and

n is from 2 to about 50.

A further class of oligonucleotide mimetic is referred to as cyclohexenyl nucleic acids (CeNA). The furanose ring normally present in an DNA/RNA molecule is replaced with a cyclohexenyl ring. CeNA DMT protected phosphoramidite monomers have been prepared and used for oligomeric compound synthesis following classical phosphoramidite chemistry. Fully modified CeNA oligomeric compounds and oligonucleotides having specific positions modified with CeNA have been prepared and studied (see Wang *et al.*, *J. Am. Chem. Soc.*, **2000**, 122, 8595-8602). In general the incorporation of CeNA monomers into a DNA chain increases its stability of a DNA/RNA hybrid. CeNA oligoadenylates formed complexes with RNA and DNA complements with similar stability to the native complexes. The study of incorporating CeNA structures into natural nucleic acid structures was shown by NMR and circular dichroism to proceed

with easy conformational adaptation. Furthermore the incorporation of CeNA into a sequence targeting RNA was stable to serum and able to activate E. Coli RNase resulting in cleavage of the target RNA strand.

5 The general formula of CeNA is shown below:



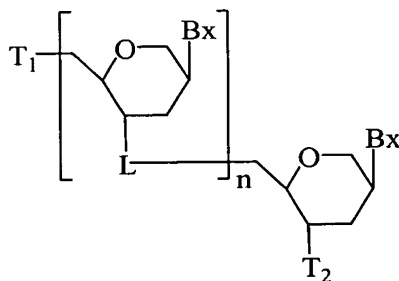
wherein

10 each Bx is a heterocyclic base moiety;

T₁ is hydroxyl or a protected hydroxyl; and

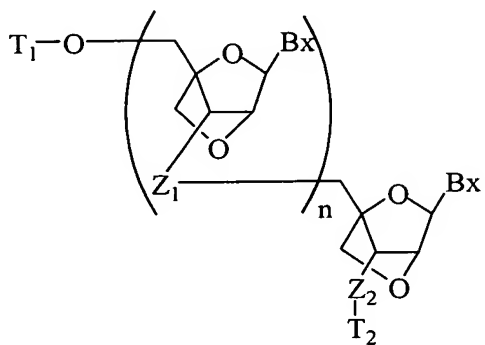
T₂ is hydroxyl or a protected hydroxyl.

Another class of oligonucleotide mimetic (anhydrohexitol nucleic acid) can be prepared from one or more anhydrohexitol nucleosides (see, Wouters and Herdewijn, *Bioorg. Med. Chem. Lett.*, **1999**, 9, 1563-1566) and would have the general formula:



A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH₂-)_n group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2 (Singh et al., *Chem. Commun.*, 1998, 4, 455-456). LNA and LNA
25 analogs display very high duplex thermal stabilities with complementary DNA and RNA (T_m = +3 to +10 C), stability

towards 3'-exonucleolytic degradation and good solubility properties. The basic structure of LNA showing the bicyclic ring system is shown below:



5

The conformations of LNAs determined by 2D NMR spectroscopy have shown that the locked orientation of the LNA nucleotides, both in single-stranded LNA and in duplexes, constrains the phosphate backbone in such a way as to introduce a higher population of the N-type conformation (Petersen et al., J. Mol. Recognit., 2000, 13, 44-53). These conformations are associated with improved stacking of the nucleobases (Wengel et al., Nucleosides Nucleotides, 1999, 18, 1365-1370).

LNA has been shown to form exceedingly stable LNA:LNA duplexes (Koshkin et al., J. Am. Chem. Soc., 1998, 120, 13252-13253). LNA:LNA hybridization was shown to be the most thermally stable nucleic acid type duplex system, and the RNA-mimicking character of LNA was established at the duplex level. Introduction of 3 LNA monomers (T or A) significantly increased melting points ($T_m = +15/+11$) toward DNA complements. The universality of LNA-mediated hybridization has been stressed by the formation of exceedingly stable LNA:LNA duplexes. The RNA-mimicking of LNA was reflected with regard to the N-type conformational restriction of the monomers and to the secondary structure of the LNA:RNA duplex.

25

LNAs also form duplexes with complementary DNA, RNA or LNA with high thermal affinities. Circular dichroism (CD) spectra show that duplexes involving fully modified LNA (esp. LNA:RNA) structurally resemble an A-form RNA:RNA duplex.

5 Nuclear magnetic resonance (NMR) examination of an LNA:DNA duplex confirmed the 3'-endo conformation of an LNA monomer. Recognition of double-stranded DNA has also been demonstrated suggesting strand invasion by LNA. Studies of mismatched sequences show that LNAs obey the Watson-Crick base pairing
10 rules with generally improved selectivity compared to the corresponding unmodified reference strands.

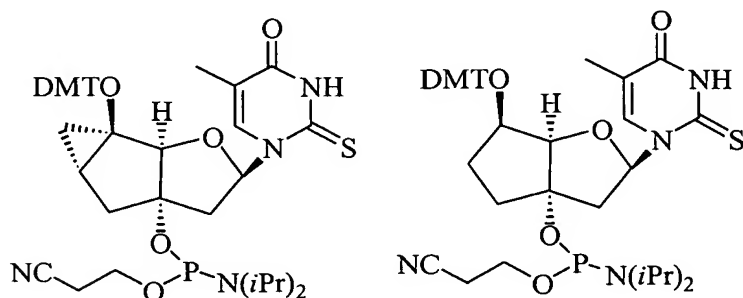
Novel types of LNA-oligomeric compounds, as well as the LNAs, are useful in a wide range of diagnostic and therapeutic applications. Among these are antisense applications, PCR
15 applications, strand-displacement oligomers, substrates for nucleic acid polymerases and generally as nucleotide based drugs. Potent and nontoxic antisense oligonucleotides containing LNAs have been described (Wahlestedt et al., Proc. Natl. Acad. Sci. U. S. A., 2000, 97, 5633-5638.) The authors
20 have demonstrated that LNAs confer several desired properties to antisense agents. LNA/DNA copolymers were not degraded readily in blood serum and cell extracts. LNA/DNA copolymers exhibited potent antisense activity in assay systems as disparate as G-protein-coupled receptor signaling in living
25 rat brain and detection of reporter genes in *Escherichia coli*. Lipofectin-mediated efficient delivery of LNA into living human breast cancer cells has also been accomplished.

The synthesis and preparation of the LNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine and
30 uracil, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin et al., Tetrahedron, 1998, 54, 3607-3630). LNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

The first analogs of LNA, phosphorothioate-LNA and 2'-
35 thio-LNAs, have also been prepared (Kumar et al., Bioorg. Med.

Chem. Lett., 1998, 8, 2219-2222). Preparation of locked nucleoside analogs containing oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (Wengel et al., PCT International Application
5 WO 98-DK393 19980914). Furthermore, synthesis of 2'-amino-LNA, a novel conformationally restricted high-affinity oligonucleotide analog with a handle has been described in the art (Singh et al., J. Org. Chem., 1998, 63, 10035-10039). In addition, 2'-Amino- and 2'-methylamino-LNA's have been
10 prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

Further oligonucleotide mimetics have been prepared to include bicyclic and tricyclic nucleoside analogs having the
15 formulas (amidite monomers shown):

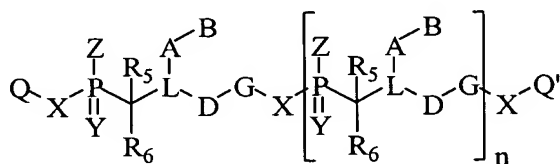


(see Steffens et al., *Helv. Chim. Acta*, **1997**, 80, 2426-2439; Steffens et al., *J. Am. Chem. Soc.*, **1999**, 121, 3249-3255; and
20 Renneberg et al., *J. Am. Chem. Soc.*, **2002**, 124, 5993-6002). These modified nucleoside analogs have been oligomerized using the phosphoramidite approach and the resulting oligomeric compounds containing tricyclic nucleoside analogs have shown increased thermal stabilities (*T_m*'s) when hybridized to DNA,
25 RNA and itself. Oligomeric compounds containing bicyclic nucleoside analogs have shown thermal stabilities approaching that of DNA duplexes.

Another class of oligonucleotide mimetic is referred to as phosphonomonoester nucleic acids incorporate a phosphorus

group in a backbone the backbone. This class of oligonucleotide mimetic is reported to have useful physical and biological and pharmacological properties in the areas of inhibiting gene expression (antisense oligonucleotides, ribozymes, sense oligonucleotides and triplex-forming oligonucleotides), as probes for the detection of nucleic acids and as auxiliaries for use in molecular biology.

The general formula (for definitions of Markush variables see: United States Patents 5,874,553 and 6,127,346 herein incorporated by reference in their entirety) is shown below.



Another oligonucleotide mimetic has been reported wherein the furanosyl ring has been replaced by a cyclobutyl moiety.

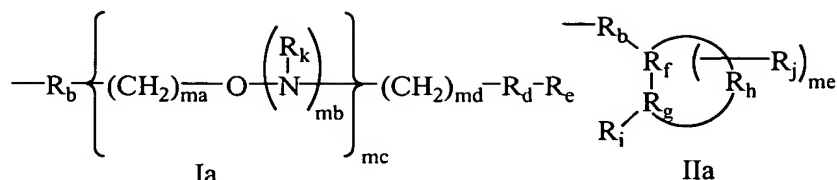
Modified sugars

Oligomeric compounds of the invention may also contain one or more substituted sugar moieties. Preferred oligomeric compounds comprise a sugar substituent group selected from: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise a sugar substituent group selected from: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for

improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, **1995**, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-aminoethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH₂-O-CH₂-N(CH₃)₂.

Other preferred sugar substituent groups include methoxy (-O-CH₃), aminopropoxy (-OCH₂CH₂CH₂NH₂), allyl (-CH₂-CH=CH₂), -O-allyl (-O-CH₂-CH=CH₂) and fluoro (F). 2'-Sugar substituent groups may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligomeric compound, particularly the 3' position of the sugar on the 3' terminal nucleoside or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligomeric compounds may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Further representative sugar substituent groups include groups of formula I_a or II_a:

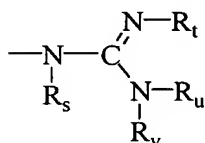


wherein:

R_b is O, S or NH;

R_d is a single bond, O, S or C(=O);

5 R_e is C₁-C₁₀ alkyl, N(R_k)(R_m), N(R_k)(R_n), N=C(R_p)(R_q),
N=C(R_p)(R_r) or has formula IIIa;



IIIa

R_p and R_q are each independently hydrogen or C₁-C₁₀ alkyl;

R_r is -R_x-R_y;

10 each R_s, R_t, R_u and R_v is, independently, hydrogen, C(O)R_w,
substituted or unsubstituted C₁-C₁₀ alkyl, substituted or
unsubstituted C₂-C₁₀ alkenyl, substituted or unsubstituted C₂-
C₁₀ alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional
15 group or a conjugate group, wherein the substituent groups are
selected from hydroxyl, amino, alkoxy, carboxy, benzyl,
phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl,
alkenyl and alkynyl;

or optionally, R_u and R_v, together form a phthalimido
moiety with the nitrogen atom to which they are attached;

20 each R_w is, independently, substituted or unsubstituted
C₁-C₁₀ alkyl, trifluoromethyl, cyanoethoxy, methoxy, ethoxy,
t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-
ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-
butyryl, phenyl or aryl;

25 R_k is hydrogen, a nitrogen protecting group or -R_x-R_y;

R_p is hydrogen, a nitrogen protecting group or -R_x-R_y;

R_x is a bond or a linking moiety;

R_y is a chemical functional group, a conjugate group or a solid support medium;

each R_m and R_n is, independently, H, a nitrogen protecting group, substituted or unsubstituted C_1 - C_{10} alkyl, substituted or unsubstituted C_2 - C_{10} alkenyl, substituted or unsubstituted C_2 - C_{10} alkynyl, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl; NH_3^+ , $N(R_u)(R_v)$, guanidino and acyl where said acyl is an acid amide or an ester;

or R_m and R_n , together, are a nitrogen protecting group, are joined in a ring structure that optionally includes an additional heteroatom selected from N and O or are a chemical functional group;

R_i is OR_z , SR_z , or $N(R_z)_2$;

each R_z is, independently, H, C_1 - C_8 alkyl, C_1 - C_8 haloalkyl, $C(=NH)N(H)R_u$, $C(=O)N(H)R_u$ or $OC(=O)N(H)R_u$;

R_f , R_g and R_h comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

R_j is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, $N(R_k)(R_m)$, OR_k , halo, SR_k or CN;

m_a is 1 to about 10;

each m_b is, independently, 0 or 1;

m_c is 0 or an integer from 1 to 10;

m_d is an integer from 1 to 10;

m_e is from 0, 1 or 2; and

provided that when m_c is 0, m_d is greater than 1.

Representative substituents groups of Formula I are disclosed in United States Patent Application Serial No.

09/130,973, filed August 7, 1998, entitled "Capped 2'-Oxyethoxy Oligonucleotides," hereby incorporated by reference in its entirety.

Representative cyclic substituent groups of Formula II are disclosed in United States Patent Application Serial No. 09/123,108, filed July 27, 1998, entitled "RNA Targeted 2'-Oligomeric compounds that are Conformationally Preorganized," hereby incorporated by reference in its entirety.

Particularly preferred sugar substituent groups include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10.

Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned United States Patent Application 09/349,040, entitled "Functionalized Oligomers", filed July 7, 1999, hereby incorporated by reference in its entirety.

Representative acetamido substituent groups are disclosed in United States Patent 6,147,200 which is hereby incorporated by reference in its entirety.

Representative dimethylaminoethoxyethyl substituent groups are disclosed in International Patent Application PCT/US99/17895, entitled "2'-O-Dimethylaminoethoxyethyl-Oligomeric compounds", filed August 6, 1999, hereby incorporated by reference in its entirety.

Modified Nucleobases/Naturally occurring nucleobases

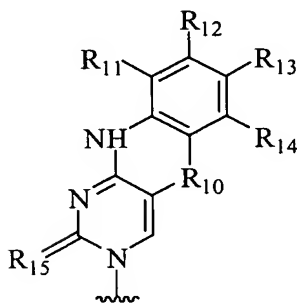
Oligomeric compounds may also include nucleobase (often referred to in the art simply as "base" or "heterocyclic base moiety") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases also referred herein as heterocyclic base moieties include other synthetic and natural nucleobases such as 5-methylcytosine (5-

me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Heterocyclic base moieties may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base

substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

In one aspect of the present invention oligomeric compounds are prepared having polycyclic heterocyclic compounds in place of one or more heterocyclic base moieties. A number of tricyclic heterocyclic compounds have been previously reported. These compounds are routinely used in antisense applications to increase the binding properties of the modified strand to a target strand. The most studied modifications are targeted to guanosines hence they have been termed G-clamps or cytidine analogs. Many of these polycyclic heterocyclic compounds have the general formula:



15

Representative cytosine analogs that make 3 hydrogen bonds with a guanosine in a second strand include 1,3-diazaphenoxazine-2-one (R₁₀ = O, R₁₁ - R₁₄ = H) [Kurchavov, et al., *Nucleosides and Nucleotides*, 1997, 16, 1837-1846], 1,3-diazaphenothiazine-2-one (R₁₀ = S, R₁₁ - R₁₄ = H), [Lin, K.-Y.; Jones, R. J.; Matteucci, M. J. Am. Chem. Soc. 1995, 117, 3873-3874] and 6,7,8,9-tetrafluoro-1,3-diazaphenoxazine-2-one (R₁₀ = O, R₁₁ - R₁₄ = F) [Wang, J.; Lin, K.-Y., Matteucci, M. Tetrahedron Lett. 1998, 39, 8385-8388]. Incorporated into oligonucleotides these base modifications were shown to hybridize with complementary guanine and the latter was also shown to hybridize with adenine and to enhance helical thermal stability by extended stacking interactions (also see U.S. Patent Application entitled "Modified Peptide Nucleic Acids"

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filed May 24, 2002, Serial number 10/155,920; and U.S. Patent Application entitled "Nuclease Resistant Chimeric Oligonucleotides" filed May 24, 2002, Serial number 10/013,295, both of which are commonly owned with this application and are herein incorporated by reference in their entirety).

Further helix-stabilizing properties have been observed when a cytosine analog/substitute has an aminoethoxy moiety attached to the rigid 1,3-diazaphenoxazine-2-one scaffold ($R_{10} =$ O, $R_{11} = -O-(CH_2)_2-NH_2$, $R_{12-14}=H$) [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. Binding studies demonstrated that a single incorporation could enhance the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with a ΔT_m of up to 18° relative to 5-methyl cytosine (dC5^{me}), which is the highest known affinity enhancement for a single modification, yet. On the other hand, the gain in helical stability does not compromise the specificity of the oligonucleotides. The T_m data indicate an even greater discrimination between the perfect match and mismatched sequences compared to dC5^{me}. It was suggested that the tethered amino group serves as an additional hydrogen bond donor to interact with the Hoogsteen face, namely the O6, of a complementary guanine thereby forming 4 hydrogen bonds. This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and additional specific hydrogen bonding.

Further tricyclic heterocyclic compounds and methods of using them that are amenable to the present invention are disclosed in United States Patent Serial Number 6,028,183, which issued on May 22, 2000, and United States Patent Serial Number 6,007,992, which issued on December 28, 1999, the contents of both are commonly assigned with this application and are incorporated herein in their entirety.

The enhanced binding affinity of the phenoxazine derivatives together with their uncompromised sequence

specificity makes them valuable nucleobase analogs for the development of more potent antisense-based drugs. In fact, promising data have been derived from in vitro experiments demonstrating that heptanucleotides containing phenoxazine
5 substitutions are capable to activate RNaseH, enhance cellular uptake and exhibit an increased antisense activity [Lin, K-Y; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. The activity enhancement was even more pronounced in case of G-clamp, as a single substitution was shown to significantly
10 improve the in vitro potency of a 20mer 2'-deoxyphosphorothioate oligonucleotides [Flanagan, W. M.; Wolf, J.J.; Olson, P.; Grant, D.; Lin, K.-Y.; Wagner, R. W.; Matteucci, M. Proc. Natl. Acad. Sci. USA, 1999, 96, 3513-3518]. Nevertheless, to optimize oligonucleotide design and
15 to better understand the impact of these heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers.

Further modified polycyclic heterocyclic compounds useful
20 as heterocyclic bases are disclosed in but not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,434,257; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091;
25 5,614,617; 5,645,985; 5,646,269; 5,750,692; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, and United States Patent Application Serial number 09/996,292 filed November 28, 2001, certain of which are commonly owned with the instant application, and each of which is herein incorporated by
30 reference.

The oligonucleotides of the present invention also include variants in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenosine, variants
35 may be produced which contain thymidine, guanosine or cytidine

at this position. This may be done at any of the positions of the oligonucleotide. Thus, a 20-mer may comprise 60 variations (20 positions x 3 alternates at each position) in which the original nucleotide is substituted with any of the three
5 alternate nucleotides. These oligonucleotides are then tested using the methods described herein to determine their ability to inhibit expression of HCV mRNA and/or HCV replication.

Conjugates

10 A further preferred substitution that can be appended to the oligomeric compounds of the invention involves the linkage of one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the resulting oligomeric compounds. In one embodiment such
15 modified oligomeric compounds are prepared by covalently attaching conjugate groups to functional groups such as hydroxyl or amino groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that
20 enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines,
25 coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties,
30 in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by
35 reference. Conjugate moieties include but are not limited to

lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1994**, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1993**, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, 259, 327-330; Svinarchuk et al., *Biochimie*, **1993**, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, 277, 923-937).

The oligomeric compounds of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepam, indomethacin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

Chimeric oligomeric compounds

It is not necessary for all positions in an oligomeric compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligomeric compound or even at a single monomeric subunit such as a nucleoside within a oligomeric compound. The present invention also includes oligomeric compounds which are chimeric oligomeric compounds. "Chimeric" oligomeric compounds or "chimeras," in the context of this invention, are oligomeric compounds that contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of a nucleic acid based oligomer.

Chimeric oligomeric compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An

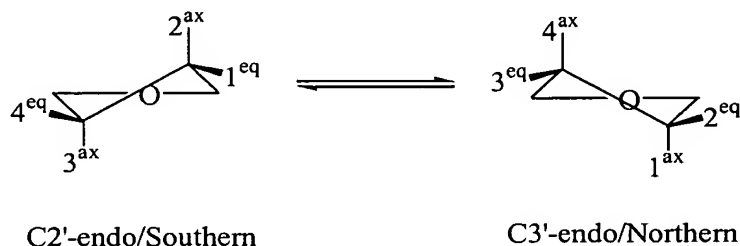
additional region of the oligomeric compound may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligomeric compounds when chimeras are used, compared to for example phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric oligomeric compounds of the invention may be formed as composite structures of two or more oligonucleotides, oligonucleotide analogs, oligonucleosides and/or oligonucleotide mimetics as described above. Such oligomeric compounds have also been referred to in the art as hybrids hemimers, gapmers or inverted gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

3'-endo modifications

In one aspect of the present invention oligomeric compounds include nucleosides synthetically modified to induce a 3'-endo sugar conformation. A nucleoside can incorporate synthetic modifications of the heterocyclic base, the sugar moiety or both to induce a desired 3'-endo sugar conformation. These modified nucleosides are used to mimic RNA like nucleosides so that particular properties of an oligomeric

compound can be enhanced while maintaining the desirable 3'-endo conformational geometry. There is an apparent preference for an RNA type duplex (A form helix, predominantly 3'-endo) as a requirement (e.g. trigger) of RNA interference which is supported in part by the fact that duplexes composed of 2'-deoxy-2'-F-nucleosides appears efficient in triggering RNAi response in the *C. elegans* system. Properties that are enhanced by using more stable 3'-endo nucleosides include but aren't limited to modulation of pharmacokinetic properties through modification of protein binding, protein off-rate, absorption and clearance; modulation of nuclease stability as well as chemical stability; modulation of the binding affinity and specificity of the oligomer (affinity and specificity for enzymes as well as for complementary sequences); and increasing efficacy of RNA cleavage. The present invention provides oligomeric triggers of RNAi having one or more nucleosides modified in such a way as to favor a C3'-endo type conformation.

Scheme 1

Nucleoside conformation is influenced by various factors including substitution at the 2', 3' or 4'-positions of the pentofuranosyl sugar. Electronegative substituents generally prefer the axial positions, while sterically demanding substituents generally prefer the equatorial positions (Principles of Nucleic Acid Structure, Wolfgang Sanger, 1984, Springer-Verlag.) Modification of the 2' position to favor the 3'-endo conformation can be achieved while maintaining the 2'-OH as a recognition element, as illustrated in Figure 2,

below (Gallo et al., Tetrahedron (2001), 57, 5707-5713. Harry-O'kuru et al., J. Org. Chem., (1997), 62(6), 1754-1759 and Tang et al., J. Org. Chem. (1999), 64, 747-754.)

Alternatively, preference for the 3'-endo conformation can be achieved by deletion of the 2'-OH as exemplified by 2'-deoxy-2'-F-nucleosides (Kawasaki et al., J. Med. Chem. (1993), 36, 831-841), which adopts the 3'-endo conformation positioning the electronegative fluorine atom in the axial position.

Other modifications of the ribose ring, for example

substitution at the 4'-position to give 4'-F modified nucleosides (Guillerm et al., Bioorganic and Medicinal Chemistry Letters (1995), 5, 1455-1460 and Owen et al., J. Org. Chem. (1976), 41, 3010-3017), or for example modification to yield methanocarba nucleoside analogs (Jacobson et al., J. Med. Chem. Lett. (2000), 43, 2196-2203 and Lee et al., Bioorganic and Medicinal Chemistry Letters (2001), 11, 1333-1337) also induce preference for the 3'-endo conformation.

Along similar lines, oligomeric triggers of RNAi response might be composed of one or more nucleosides modified in such a way that conformation is locked into a C3'-endo type conformation, i.e. Locked Nucleic Acid (LNA, Singh et al, Chem. Commun. (1998), 4, 455-456), and ethylene bridged Nucleic Acids (ENA, Morita et al, Bioorganic & Medicinal Chemistry Letters (2002), 12, 73-76.) Examples of modified nucleosides amenable to the present invention are shown below in Table I. These examples are meant to be representative and not exhaustive.

modified oligonucleotides of the present invention. The synthesis of numerous of the modified nucleosides amenable to the present invention are known in the art (see for example, Chemistry of Nucleosides and Nucleotides Vol 1-3, ed. Leroy B. Townsend, 1988, Plenum press., and the examples section below.) Nucleosides known to be inhibitors/substrates for RNA dependent RNA polymerases (for example HCV NS5B

In one aspect, the present invention is directed to oligonucleotides that are prepared having enhanced properties compared to native RNA against nucleic acid targets. A target is identified and an oligonucleotide is selected having an effective length and sequence that is complementary to a portion of the target sequence. Each nucleoside of the selected sequence is scrutinized for possible enhancing modifications. A preferred modification would be the replacement of one or more RNA nucleosides with nucleosides that have the same 3'-endo conformational geometry. Such modifications can enhance chemical and nuclease stability relative to native RNA while at the same time being much cheaper and easier to synthesize and/or incorporate into an oligonucleotide. The selected sequence can be further divided into regions and the nucleosides of each region evaluated for enhancing modifications that can be the result of a chimeric configuration. Consideration is also given to the 5' and 3'-termini as there are often advantageous modifications that can be made to one or more of the terminal nucleosides. The oligomeric compounds of the present invention include at least one 5'-modified phosphate group on a single strand or on at least one 5'-position of a double stranded sequence or sequences. Further modifications are also considered such as internucleoside linkages, conjugate groups, substitute sugars or bases, substitution of one or more nucleosides with nucleoside mimetics and any other modification that can enhance the selected sequence for its intended target. The terms used to describe the conformational geometry of homoduplex nucleic

acids are "A Form" for RNA and "B Form" for DNA. The respective conformational geometry for RNA and DNA duplexes was determined from X-ray diffraction analysis of nucleic acid fibers (Arnott and Hukins, *Biochem. Biophys. Res. Comm.*, 1970, 47, 1504.) In general, RNA:RNA duplexes are more stable and have higher melting temperatures (T_m 's) than DNA:DNA duplexes (Sanger et al., *Principles of Nucleic Acid Structure*, 1984, Springer-Verlag; New York, NY.; Lesnik et al., *Biochemistry*, 1995, 34, 10807-10815; Conte et al., *Nucleic Acids Res.*, 1997, 25, 2627-2634). The increased stability of RNA has been attributed to several structural features, most notably the improved base stacking interactions that result from an A-form geometry (Searle et al., *Nucleic Acids Res.*, 1993, 21, 2051-2056). The presence of the 2' hydroxyl in RNA biases the sugar toward a C3' endo pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. In addition, the 2' hydroxyl groups of RNA can form a network of water mediated hydrogen bonds that help stabilize the RNA duplex (Egli et al., *Biochemistry*, 1996, 35, 8489-8494). On the other hand, deoxy nucleic acids prefer a C2' endo sugar pucker, i.e., also known as Southern pucker, which is thought to impart a less stable B-form geometry (Sanger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, NY). As used herein, B-form geometry is inclusive of both C2'-endo pucker and O4'-endo pucker. This is consistent with Berger, et. al., *Nucleic Acids Research*, 1998, 26, 2473-2480, who pointed out that in considering the furanose conformations which give rise to B-form duplexes consideration should also be given to a O4'-endo pucker contribution.

DNA:RNA hybrid duplexes, however, are usually less stable than pure RNA:RNA duplexes, and depending on their sequence may be either more or less stable than DNA:DNA duplexes (Searle et al., *Nucleic Acids Res.*, 1993, 21, 2051-2056). The structure of a hybrid duplex is intermediate between A- and B-

form geometries, which may result in poor stacking interactions (Lane et al., *Eur. J. Biochem.*, **1993**, 215, 297-306; Fedoroff et al., *J. Mol. Biol.*, **1993**, 233, 509-523; Gonzalez et al., *Biochemistry*, **1995**, 34, 4969-4982; Horton et al., *J. Mol. Biol.*, **1996**, 264, 521-533). The stability of the duplex formed between a target RNA and a synthetic sequence is central to therapies such as but not limited to antisense and RNA interference as these mechanisms require the binding of a synthetic oligonucleotide strand to an RNA target strand. In the case of antisense, effective inhibition of the mRNA requires that the antisense DNA have a very high binding affinity with the mRNA. Otherwise the desired interaction between the synthetic oligonucleotide strand and target mRNA strand will occur infrequently, resulting in decreased efficacy.

One routinely used method of modifying the sugar pucker is the substitution of the sugar at the 2'-position with a substituent group that influences the sugar geometry. The influence on ring conformation is dependant on the nature of the substituent at the 2'-position. A number of different substituents have been studied to determine their sugar pucker effect. For example, 2'-halogens have been studied showing that the 2'-fluoro derivative exhibits the largest population (65%) of the C3'-endo form, and the 2'-iodo exhibits the lowest population (7%). The populations of adenosine (2'-OH) versus deoxyadenosine (2'-H) are 36% and 19%, respectively. Furthermore, the effect of the 2'-fluoro group of adenosine dimers (2'-deoxy-2'-fluoroadenosine - 2'-deoxy-2'-fluoro-adenosine) is further correlated to the stabilization of the stacked conformation.

As expected, the relative duplex stability can be enhanced by replacement of 2'-OH groups with 2'-F groups thereby increasing the C3'-endo population. It is assumed that the highly polar nature of the 2'-F bond and the extreme preference for C3'-endo puckering may stabilize the stacked

conformation in an A-form duplex. Data from UV hypochromicity, circular dichroism, and ^1H NMR also indicate that the degree of stacking decreases as the electronegativity of the halo substituent decreases. Furthermore, steric bulk at the 2'-position of the sugar moiety is better accommodated in an A-form duplex than a B-form duplex. Thus, a 2'-substituent on the 3'-terminus of a dinucleoside monophosphate is thought to exert a number of effects on the stacking conformation: steric repulsion, furanose puckering preference, electrostatic repulsion, hydrophobic attraction, and hydrogen bonding capabilities. These substituent effects are thought to be determined by the molecular size, electronegativity, and hydrophobicity of the substituent. Melting temperatures of complementary strands is also increased with the 2'-substituted adenosine diphosphates. It is not clear whether the 3'-endo preference of the conformation or the presence of the substituent is responsible for the increased binding. However, greater overlap of adjacent bases (stacking) can be achieved with the 3'-endo conformation.

One synthetic 2'-modification that imparts increased nuclease resistance and a very high binding affinity to nucleotides is the 2-methoxyethoxy (2'-MOE, 2'-OCH₂CH₂OCH₃) side chain (Baker et al., *J. Biol. Chem.*, **1997**, 272, 11944-12000). One of the immediate advantages of the 2'-MOE substitution is the improvement in binding affinity, which is greater than many similar 2' modifications such as O-methyl, O-propyl, and O-aminopropyl. Oligonucleotides having the 2'-O-methoxyethyl substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P., *Helv. Chim. Acta*, **1995**, 78, 486-504; Altmann et al., *Chimia*, **1996**, 50, 168-176; Altmann et al., *Biochem. Soc. Trans.*, **1996**, 24, 630-637; and Altmann et al., *Nucleosides Nucleotides*, 1997, **16**, 917-926). Relative to DNA, the oligonucleotides having the 2'-MOE

modification displayed improved RNA affinity and higher nuclease resistance. Chimeric oligonucleotides having 2'-MOE substituents in the wing nucleosides and an internal region of deoxy-phosphorothioate nucleotides (also termed a gapped
5 oligonucleotide or gapmer) have shown effective reduction in the growth of tumors in animal models at low doses. 2'-MOE substituted oligonucleotides have also shown outstanding promise as antisense agents in several disease states. One such MOE substituted oligonucleotide is presently being
10 investigated in clinical trials for the treatment of CMV retinitis.

Chemistries Defined

Unless otherwise defined herein, alkyl means C₁-C₁₂, preferably C₁-C₈, and more preferably C₁-C₆, straight or (where
15 possible) branched chain aliphatic hydrocarbyl.

Unless otherwise defined herein, heteroalkyl means C₁-C₁₂, preferably C₁-C₈, and more preferably C₁-C₆, straight or (where possible) branched chain aliphatic hydrocarbyl containing at
20 least one, and preferably about 1 to about 3, hetero atoms in the chain, including the terminal portion of the chain. Preferred heteroatoms include N, O and S.

Unless otherwise defined herein, cycloalkyl means C₃-C₁₂, preferably C₃-C₈, and more preferably C₃-C₆, aliphatic hydrocarbyl ring.

25 Unless otherwise defined herein, alkenyl means C₂-C₁₂, preferably C₂-C₈, and more preferably C₂-C₆ alkenyl, which may be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon double bond.

Unless otherwise defined herein, alkynyl means C₂-C₁₂, preferably C₂-C₈, and more preferably C₂-C₆ alkynyl, which may
30 be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon triple bond.

Unless otherwise defined herein, heterocycloalkyl means a ring moiety containing at least three ring members, at least
35 one of which is carbon, and of which 1, 2 or three ring

members are other than carbon. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred heterocycloalkyl groups include morpholino, thiomorpholino, piperidinyl, piperazinyl, homopiperidinyl, homopiperazinyl, homomorpholino, homothiomorpholino, pyrrolodiny, tetrahydrooxazolyl, tetrahydroimidazolyl, tetrahydrothiazolyl, tetrahydroisoxazolyl, tetrahydropyrrazolyl, furanyl, pyranal, and tetrahydroisothiazolyl.

Unless otherwise defined herein, aryl means any hydrocarbon ring structure containing at least one aryl ring. Preferred aryl rings have about 6 to about 20 ring carbons. Especially preferred aryl rings include phenyl, naphthyl, anthracenyl, and phenanthrenyl.

Unless otherwise defined herein, hetaryl means a ring moiety containing at least one fully unsaturated ring, the ring consisting of carbon and non-carbon atoms. Preferably the ring system contains about 1 to about 4 rings. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred hetaryl moieties include pyrazolyl, thiophenyl, pyridyl, imidazolyl, tetrazolyl, pyridyl, pyrimidinyl, purinyl, quinazolinyl, quinoxalinyl, benzimidazolyl, benzothiophenyl, etc.

Unless otherwise defined herein, where a moiety is defined as a compound moiety, such as hetarylalkyl (hetaryl and alkyl), aralkyl (aryl and alkyl), etc., each of the sub-moieties is as defined herein.

Unless otherwise defined herein, an electron withdrawing group is a group, such as the cyano or isocyanato group that draws electronic charge away from the carbon to which it is

attached. Other electron withdrawing groups of note include those whose electronegativities exceed that of carbon, for example halogen, nitro, or phenyl substituted in the ortho- or para-position with one or more cyano, isothiocyanato, nitro or halo groups.

Unless otherwise defined herein, the terms halogen and halo have their ordinary meanings. Preferred halo (halogen) substituents are Cl, Br, and I.

The aforementioned optional substituents are, unless otherwise herein defined, suitable substituents depending upon desired properties. Included are halogens (Cl, Br, I), alkyl, alkenyl, and alkynyl moieties, NO₂, NH₃ (substituted and unsubstituted), acid moieties (e.g. -CO₂H, -OSO₃H₂, etc.), heterocycloalkyl moieties, hetaryl moieties, aryl moieties, etc. In all the preceding formulae, the squiggle (~) indicates a bond to an oxygen or sulfur of the 5'-phosphate.

Phosphate protecting groups include those described in US Patents No. US 5,760,209, US 5,614,621, US 6,051,699, US 6,020,475, US 6,326,478, US 6,169,177, US 6,121,437, US 6,465,628 each of which is expressly incorporated herein by reference in its entirety.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and 2'-alkoxy or 2'-alkoxyalkoxy derivatives, including 2'-O-methoxyethyl oligonucleotides (Martin, P., *Helv. Chim. Acta* **1995**, 78, 486-504). It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-

modified amidites and/or CPG (available from Glen Research, Sterling, VA) to synthesize fluorescently labeled, biotinylated or other conjugated oligonucleotides.

The antisense compounds of the present invention include
5 bioequivalent compounds, including pharmaceutically acceptable salts and prodrugs. This is intended to encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or
10 indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of the nucleic acids of the invention and prodrugs of such nucleic acids. Pharmaceutically acceptable salts are physiologically and
15 pharmaceutically acceptable salts of the nucleic acids of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.* **1977**, 66, 1-19).

20 For oligonucleotides, examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with
25 inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; 8 salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid,
30 ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as
35 chlorine, bromine, and iodine.

The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a Aprodug® form. The term Aprodug® indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or oligonucleotides of the invention are prepared as SATE [(5-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510.

For therapeutic or prophylactic treatment, oligonucleotides are administered in accordance with this invention. Oligonucleotide compounds of the invention may be formulated in a pharmaceutical composition, which may include pharmaceutically acceptable carriers, thickeners, diluents, buffers, preservatives, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients and the like in addition to the oligonucleotide. Such compositions and formulations are comprehended by the present invention.

Pharmaceutical compositions comprising the oligonucleotides of the present invention may include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems 1991, 8, 91-192; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems 1990, 7, 1-33). One or more penetration enhancers from one or more of these broad categories may be included. Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic

acid, dicaprates, tricaprates, recinleates, monoolein (a.k.a. 1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprates, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* **1990**, 7, 1; El-Hariri et al., *J. Pharm. Pharmacol.* **1992** 44, 651-654).

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, **1996**, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations.

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* **1990**, 7, 1-33; Buur et al., *J. Control Rel.* **1990**, 14, 43-51). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee et al., *Critical Reviews in Therapeutic Drug*

Carrier Systems 1991, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., *J. Pharm. Pharmacol.* 1988, 40, 252-257).

Non-surfactants include, for example, unsaturated cyclic
5 ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives
(Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems* 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and
phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.* 1987,
10 39, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the
15 bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result
20 in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. In contrast to a carrier compound, a "pharmaceutically acceptable
25 carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind
30 so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinyl-
35 pyrrolidone or hydroxypropyl methylcellulose, etc.); fillers

(e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); or wetting agents (e.g., sodium starch glycolate, etc.). Sustained release oral sodium lauryl sulphate, etc.).

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceuticals, local anesthetics or anti-inflammatory agents, astringents, opacifiers, thickening agents, preservatives, antioxidants, such as dyes, flavoring agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the oligonucleotides of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the oligonucleotides and/or to target the oligonucleotides to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure.

A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layers made up of lipids arranged in a bilayer configuration (see, generally,

5 Chonn et al., *Current Op. Biotech.* **1995**, 6, 698-708).

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic,
10 vaginal, rectal, intranasal, epidermal, and transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intracranial, e.g., intrathecal or
15 intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops,
20 suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or
25 granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral administration may include
30 sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. In some cases it may be more effective to treat a patient with an oligonucleotide of the invention in conjunction with other traditional therapeutic modalities in order to increase the efficacy of a
35 treatment regimen. In the context of the invention, the term

"treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. For example, a patient may be treated with conventional chemotherapeutic agents such as those used for tumor and cancer treatment.

5 When used with the compounds of the invention, such chemotherapeutic agents may be used individually, sequentially, or in combination with one or more other such chemotherapeutic agents.

10 The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the
15 disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative
20 potency of individual oligonucleotides, and can generally be estimated based on EC₅₀s found to be effective *in vitro* and in *in vivo* animal models. In general, dosage is from 0.01 μ g to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20
25 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to
30 prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μ g to 100 g per kg of body weight, once or more daily, to once every 20 years.

Thus, in the context of this invention, by
35 "therapeutically effective amount" is meant the amount of the

compound which is required to have a therapeutic effect on the treated individual. This amount, which will be apparent to the skilled artisan, will depend upon the age and weight of the individual, the type of disease to be treated, perhaps
5 even the gender of the individual, and other factors which are routinely taken into consideration when designing a drug treatment. A therapeutic effect is assessed in the individual by measuring the effect of the compound on the disease state in the animal.

10 The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

EXAMPLE 1

15 Synthesis of Oligonucleotides

Unmodified oligodeoxynucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. β -cyanoethyl-diisopropyl-phosphoramidites are
20 purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of ^3H -1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation
25 cycle wait step was increased to 68 seconds and was followed by the capping step. Cytosines may be 5-methyl cytosines. (5-methyl deoxycytidine phosphoramidites available from Glen Research, Sterling, VA or Amersham Pharmacia Biotech, Piscataway, NJ)

30 2'-methoxy oligonucleotides are synthesized using 2'-methoxy β -cyanoethyl-diisopropyl-phosphoramidites (Chemgenes, Needham, MA) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base is increased to 360 seconds. Other 2'-
35 alkoxy oligonucleotides are synthesized by a modification of

this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, VA.

2'-fluoro oligonucleotides are synthesized as described in Kawasaki et al. (*J. Med. Chem.* **1993**, 36, 831-841).

5 Briefly, the protected nucleoside N⁶-benzoyl-2'-deoxy-2'-fluoroadenosine is synthesized utilizing commercially available 9-β-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-α-fluoro atom is introduced by a S_N2-displacement of a 2'-β-O-triflyl
10 group. Thus N⁶-benzoyl-9-β-D-arabinofuranosyladenine is selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N⁶-benzoyl groups is accomplished using standard methodologies. Standard methods are also used to obtain the
15 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine is accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-β-D-arabinofuranosylguanine as starting material, and
20 conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group is followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation is followed by
25 treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies are used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

Synthesis of 2'-deoxy-2'-fluorouridine is accomplished by the modification of a known procedure in which 2, 2'-
30 anhydro-1-β-D-arabinofuranosyluracil is treated with 70% hydrogen fluoride-pyridine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'-phosphoramidites.

2'-deoxy-2'-fluorocytidine is synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection
35 to give N⁴-benzoyl-2'-deoxy-2'-fluorocytidine. Standard

procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-(2-methoxyethyl)-modified amidites were synthesized according to Martin, P. (*Helv. Chim. Acta* **1995**, 78, 486-506).

5 For ease of synthesis, the last nucleotide may be a deoxynucleotide. 2'-O-CH₂CH₂OCH₃-cytosines may be 5-methyl cytosines.

Synthesis of 5-Methyl cytosine monomers:

10 2,2'-Anhydro[1-(β-D-arabinofuranosyl)-5-methyluridine]:

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated
15 to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether
20 was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60EC at 1 mm Hg for 24 hours) to give a solid which was crushed to a light tan powder
25 (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine:

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2
30 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160EC. After heating for 48 hours at 155-160EC, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The
35 residue was suspended in hot acetone (1 L). The insoluble

salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH.

5 The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

10 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture
15 stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was
20 evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL of saturated NaCl. The organic phase was dried over Na₂SO₄, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg
25 silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et₃NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

30

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture
35 prepared from 562 mL of DMF and 188 mL of pyridine) and acetic

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anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35EC. The residue was dissolved in CHCl_3 (800 mL) and extracted with 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl_3 . The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

15 3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5EC and stirred for 0.5 hours using an overhead stirrer. POCl_3 was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10EC, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO_3 and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room
5 temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours
10 (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

15

 N^4 -Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic
20 anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl_3 (700 mL) and extracted with saturated NaHCO_3
25 (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO_4 and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the
30 title compound.

 N^4 -Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:

N^4 -Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L).
35

Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the
5 reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO₃ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were combined, dried over MgSO₄ and concentrated. The residue obtained was chromatographed on a
10 1.5 kg silica column using EtOAc\Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published
15 methods (Sanghvi et al., *Nucl. Acids Res.* **1993**, 21, 3197-3203) using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

Oligonucleotides having methylene(methylimino) (MMI) backbones were synthesized according to U.S. Patent 5,378,825,
20 which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. For ease of synthesis, various nucleoside dimers containing MMI linkages were synthesized and incorporated into oligonucleotides. Other nitrogen-containing backbones are synthesized according to WO
25 92/20823 which is also coassigned to the assignee of the present invention and incorporated herein in its entirety.

Oligonucleotides having amide backbones are synthesized according to De Mesmaeker et al. (*Acc. Chem. Res.* **1995**, 28, 366-374). The amide moiety is readily accessible by simple
30 and well-known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. Patent 5,034,506 (Summerton and
35 Weller).

Peptide-nucleic acid (PNA) oligomers are synthesized according to P.E. Nielsen et al. (*Science* **1991**, 254, 1497-1500). After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ³¹P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al. (*J. Biol. Chem.* **1991**, 266, 18162). Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

EXAMPLE 2

Human TNF- α Oligodeoxynucleotide Sequences

Antisense oligonucleotides were designed to target human TNF- α . Target sequence data are from the TNF- α cDNA sequence published by Nedwin, G.E. et al. (*Nucleic Acids Res.* **1985**, 13, 6361-6373); Genbank accession number X02910, provided herein as SEQ ID NO: 1. Oligodeoxynucleotides were synthesized primarily with phosphorothioate linkages. Oligonucleotide sequences are shown in Table 1. Oligonucleotide 14640 (SEQ ID NO. 2) is a published TNF- α antisense oligodeoxynucleotide targeted to the start site of the TNF- α gene (Hartmann, G., et al., *Antisense Nucleic Acid Drug Dev.*, **1996**, 6, 291-299). Oligonucleotide 2302 (SEQ ID NO. 41) is an antisense oligodeoxynucleotide targeted to the human intracellular adhesion molecule-1 (ICAM-1) and was used as an unrelated (negative) target control. Oligonucleotide 13664 (SEQ ID NO. 42) is an antisense oligodeoxynucleotide targeted to the

Herpes Simplex Virus type 1 and was used as an unrelated target control.

NeoHK cells, human neonatal foreskin keratinocytes (obtained from Cascade Biologicals, Inc., Portland, OR) were
5 cultured in Keratinocyte medium containing the supplied growth factors (Life Technologies, Rockville, MD).

At assay time, the cells were between 70% and 90% confluent. The cells were incubated in the presence of Keratinocyte medium, without the supplied growth factors
10 added, and the oligonucleotide formulated in LIPOFECTIN7 (Life Technologies), a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl phosphotidylethanolamine (DOPE) in membrane filtered water.
15 For an initial screen, the oligonucleotide concentration was 300 nM in 9 µg/mL LIPOFECTIN7. Treatment was for four hours. After treatment, the medium was removed and the cells were further incubated in Keratinocyte medium containing the supplied growth factors and 100 nM phorbol 12-myristate 13-
20 acetate (PMA, Sigma, St. Louis, MO). mRNA was analyzed 2 hours post-induction with PMA. Protein levels were analyzed 12 to 20 hours post-induction.

Total mRNA was isolated using the RNEASY7 Mini Kit (Qiagen, Valencia, CA; similar kits from other manufacturers
25 may also be used), separated on a 1% agarose gel, transferred to HYBONDTM-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ), a positively charged nylon membrane, and probed. A TNF-α probe consisted of the 505 bp EcoRI-HindIII fragment from BBG 18 (R&D Systems, Minneapolis, MN), a plasmid
30 containing human TNF-α cDNA. A glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe consisted of the 1.06 kb HindIII fragment from pHcGAP (American Type Culture Collection, Manassas, VA), a plasmid containing human G3PDH cDNA. The restriction fragments were purified from low-melting
35 temperature agarose, as described in Maniatis, T., et al.,

Molecular Cloning: A Laboratory Manual, 1989 and labeled with REDIVUE™ ³²P-dCTP (Amersham Pharmacia Biotech, Piscataway, NJ) and PRIME-A-GENE7 labeling kit (Promega, Madison, WI). mRNA was quantitated by a PhosphoImager (Molecular Dynamics,

- 5 Sunnyvale, CA). Secreted TNF- α protein levels were measured using a human TNF- α ELIZA kit (R&D Systems, Minneapolis, MN or Genzyme, Cambridge, MA).

10

TABLE 1

Nucleotide Sequences of Human TNF- α Phosphorothioate Oligodeoxynucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
14640	CATGCTTTT <u>C</u> AGTGCTCAT	2	0796-0813	AUG
14641	TGAGGGAGC <u>G</u> TCTGCTGGCT	3	0615-0634	5'-UTR
14642	GTGCTCATGGTGT <u>C</u> CTTTCC	4	0784-0803	AUG
14643	TAATCACAAGTGCAAACATA	5	3038-3057	3'-UTR
14644	TACCCCGGTCTCCCAAATAA	6	3101-3120	3'-UTR
14810	GTGCTCATGGTGTCTTTCC	4	0784-0803	AUG
14811	AGCACCGCCTGGAGCCCT	7	0869-0886	coding
14812	GCTGAGGAACAAGCACCGCC	8	0878-0897	coding
14813	AGGCAGAAGAGCGTGGTGGC	9	0925-0944	coding
14814	AAAGTGCAGCAGGCAGAAGA	10	0935-0954	coding
14815	TTAGAGAGAGGTCCCTGG	11	1593-1610	coding
14816	TGACTGCCTGGGCCAGAG	12	1617-1634	junction
14817	GGGTTCGAGAAGATGATC	13	1822-1839	junction

14818	GGGCTACAGGCTTGTCACCTC	14	1841-1860	coding
14820	CCCCTCAGCTTGAGGGTTTG	15	2171-2190	junction
14821	CCATTGGCCAGGAGGGCATT	16	2218-2237	coding
14822	ACCACCAGCTGGTTATCTCT	17	2248-2267	coding
14823	CTGGGAGTAGATGAGGTACA	18	2282-2301	coding
14824	CCCTTGAAGAGGACCTGGGA	19	2296-2315	coding
14825	GGTGTGGGTGAGGAGCACAT	20	2336-2355	coding
14826	GTCTGGTAGGAGACGGCGAT	21	2365-2384	coding
14827	GCAGAGAGGAGGTTGACCTT	22	2386-2405	coding
14828	GCTTGGCCTCAGCCCCCTCT	23	2436-2455	coding
14829	CCTCCCAGATAGATGGGCTC	24	2464-2483	coding
14830	CCCTTCTCCAGCTGGAAGAC	25	2485-2504	coding
14831	ATCTCAGCGCTGAGTCGGTC	26	2506-2525	coding
14832	TCGAGATAGTCGGGCCGATT	27	2527-2546	coding
14833	AAGTAGACCTGCCCAGACTC	28	2554-2573	coding
14834	GGATGTTTCGTCCTCCTCACA	29	2588-2607	STOP
14835	ACCCTAAGCCCCCAATTCTC	30	2689-2708	3'-UTR
14836	CCACACATTCCTGAATCCCA	31	2758-2777	3'-UTR
14837	AGGCCCCAGTGAGTTCTGGA	32	2825-2844	3'-UTR
14838	GTCTCCAGATTCCAGATGTC	33	2860-2879	3'-UTR
14839	CTCAAGTCCTGCAGCATTCT	34	2902-2921	3'-UTR
14840	TGGGTCCCCCAGGATACCCC	35	3115-3134	3'-UTR
14841	ACGGAAACATGTCTGAGCC	36	3151-3170	3'-UTR
14842	CTCCGTTTTTCACGGAAAACA	37	3161-3180	3'-UTR

14843	GCCTATTGTTTCAGCTCCGTT	38	3174-3193	3'-UTR
14844	GGTCACCAAATCAGCATTGT	39	3272-3292	3'-UTR
14845	GAGGCTCAGCAATGAGTGAC	40	3297-3316	3'-UTR
2302	G <u>CCCAAGCTGGCATCCGTCA</u>	41	target control	
13664	GCCGAGGTCCATGTCGTACGC	42	target control	

¹ "C" residues are 5-methyl-cytosines except "C" residues are unmodified cytidines; all linkages are phosphorothioate linkages.

5

²Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

10 Results are shown in Table 2. Oligonucleotides 14828 (SEQ ID NO. 23), 14829 (SEQ ID NO. 24), 14832 (SEQ ID NO. 27), 14833 (SEQ ID NO. 28), 14834 (SEQ ID NO. 29), 14835 (SEQ ID NO. 30), 14836 (SEQ ID NO. 31), 14839 (SEQ ID NO. 34), 14840 (SEQ ID NO. 35), and 14844 (SEQ ID NO. 39) inhibited TNF- α expression by approximately 50% or more. Oligonucleotides 14828 (SEQ ID NO. 23), 14834 (SEQ ID NO. 29), and 14840 (SEQ ID NO. 35) gave better than 70% inhibition.

15

TABLE 2

20

Inhibition of Human TNF- α mRNA Expression by Phosphorothioate Oligodeoxynucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
basal	---	---	16%	---
induced	---	---	100%	0%
13664	42	control	140%	---
14640	2	AUG	61%	39%

14641	3	5'-UTR	95%	5%
14642	4	AUG	131%	---
14810	4	AUG	111%	---
14815	11	coding	85%	15%
14816	12	junction	106%	---
14817	13	junction	97%	3%
14818	14	coding	64%	36%
14820	15	junction	111%	---
14821	16	coding	91%	9%
14822	17	coding	57%	43%
14827	22	coding	67%	33%
14828	23	coding	27%	73%
14829	24	coding	33%	67%
14830	25	coding	71%	29%
14831	26	coding	62%	38%
14832	27	coding	40%	60%
14833	28	coding	43%	57%
14834	29	STOP	26%	74%
14835	30	3'-UTR	32%	68%
14836	31	3'-UTR	40%	60%
14837	32	3'-UTR	106%	---
14838	33	3'-UTR	70%	30%
14839	34	5'-UTR	49%	51%
14840	35	3'-UTR	28%	72%

14841	36	3'-UTR	60%	40%
14842	37	3'-UTR	164%	---
14843	38	3'-UTR	67%	33%
14844	39	3'-UTR	46%	54%
14845	40	3'-UTR	65%	35%

EXAMPLE 3**Dose response of antisense phosphorothioate****5 oligodeoxynucleotide effects on human TNF- α mRNA levels in
NeoHK cells**

Four of the more active oligonucleotides from the initial screen were chosen for dose response assays. These include oligonucleotides 14828 (SEQ ID NO. 23), 14833 (SEQ ID
10 NO. 28), 14834 (SEQ ID NO. 29) and 14839 (SEQ ID NO. 34). NeoHK cells were grown, treated and processed as described in Example 2. LIPOFECTIN⁷ was added at a ratio of 3 μ g/mL per 100 nM of oligonucleotide. The control included LIPOFECTIN⁷ at a concentration of 9 μ g/mL. The effect of the TNF- α
15 antisense oligonucleotides was normalized to the non-specific target control. Results are shown in Table 3. Each oligonucleotide showed a dose response effect with maximal inhibition greater than 70%. Oligonucleotides 14828 (SEQ ID NO. 23) had an IC₅₀ of approximately 185 nM. Oligonucleotides
20 14833 (SEQ ID NO. 28) had an IC₅₀ of approximately 150 nM. Oligonucleotides 14834 (SEQ ID NO. 29) and 14839 (SEQ ID NO. 34) had an IC₅₀ of approximately 140 nM.

TABLE 3

Dose Response of NeoHK Cells to TNF- α
Antisense Phosphorothioate Oligodeoxynucleotides (ASOs)

5

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
2302	41	control	25 nM	100%	---
"	"	"	50 nM	100%	---
"	"	"	100 nM	100%	---
"	"	"	200 nM	100%	---
"	"	"	300 nM	100%	---
14828	23	coding	25 nM	122%	---
"	"	"	50 nM	97%	3%
"	"	"	100 nM	96%	4%
"	"	"	200 nM	40%	60%
"	"	"	300 nM	22%	78%
14833	28	coding	25 nM	89%	11%
"	"	"	50 nM	8%	22%
"	"	"	100 nM	64%	36%
"	"	"	200 nM	36%	64%
"	"	"	300 nM	25%	75%
14834	29	STOP	25 nM	94%	6%
"	"	"	50 nM	69%	31%
"	"	"	100 nM	65%	35%
"	"	"	200 nM	26%	74%
"	"	"	300 nM	11%	89%
14839	34	3'-UTR	25 nM	140%	---
"	"	"	50 nM	112%	---
"	"	"	100 nM	65%	35%
"	"	"	200 nM	29%	71%
"	"	"	300 nM	22%	78%

EXAMPLE 4

Design and Testing of Chimeric (deoxy gapped) 2'-O-methoxyethyl TNF- α Antisense Oligonucleotides on TNF- α Levels in NeoHK Cells

10

Oligonucleotides having SEQ ID NO: 28 and SEQ ID NO: 29 were synthesized as uniformly phosphorothioate or mixed phosphorothioate/phosphodiester chimeric oligonucleotides

having variable regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and deoxynucleotides. The sequences and the oligonucleotide chemistries are shown in Table 4. All 2'-MOE cytosines were 5-methyl-cytosines.

5 Dose response experiments, as discussed in Example 3, were performed using these chimeric oligonucleotides. The effect of the TNF- α antisense oligonucleotides was normalized to the non-specific target control. Results are shown in Table 5. The activities of the chimeric oligonucleotides
10 tested were comparable to the parent phosphorothioate oligonucleotide.

TABLE 4
Nucleotide Sequences of TNF- α Chimeric
(deoxy gapped) 2'-O-methoxyethyl Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE (5' -> 3') ¹	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
14833	AsAsGsTsAsGsAsCsCsTsGsCsCsCsAsGsAsCsTsC	28	2554-2573	coding
16467	AoAoGoToAsGsAsCsCsTsGsCsCsCsAsGoAoCoToC	28	2554-2573	coding
16468	AsAsGsTsAsGsAsCsCsTsGsCsCsCsAsGsAsCsTsC	28	2554-2573	coding
16469	AsAsGsTsAsGsAsCsCsTsGsCsCsCsAsGsAsCsTsC	28	2554-2573	coding
16470	AsAsGsTsAsGsAsCsCsTsGs CsCsCsAsGsAsCsTsC	28	2554-2573	coding
16471	AsAsGsTsAsGsAsCsCsTsGsCsCsCsAsGsAsCsTsC	28	2554-2573	coding
14834	GsGsAsTsGsTsTsCsGsTsCsCsTsCsCsTsCsAsCsA	29	2588-2607	STOP
16472	GoGoAoToGsTsTsCsGsTsCsCsTsCsCsToCoAoCoA	29	2588-2607	STOP
16473	GsGsAsTsGsTsTsCsGsTsCsCsTsCsCsTsCsAsCsA	29	2588-2607	STOP
16474	GsGsAsTsGsTsTsCsGsTsCsCsTsCsCsTsCsAsCsA	29	2588-2607	STOP
16475	GsGsAsTsGsTsTsCsGsTsCsCsTsCsCsTsCsAsCsA	29	2588-2607	STOP
16476	GsGsAsTsGsTsTsCsGsTsCs CsTsCsCsTsCsAsCsA	29	2588-2607	STOP

¹Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines are 5-methyl-cytidines; "s" linkages are phosphorothioate linkages, "o" linkages are phosphodiester linkages.

² Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

TABLE 5

Dose Response of NeoHK Cells to TNF- α
Chimeric (deoxy gapped) 2'-O-methoxyethyl Antisense
Oligonucleotides

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
13664	42	Control	50 nM	100%	---
"	"	"	100 nM	100%	---
"	"	"	200 nM	100%	---
"	"	"	300 nM	100%	---
14833	28	Coding	50 nM	69%	31%
"	"	"	100 nM	64%	36%
"	"	"	200 nM	56%	44%
"	"	"	300 nM	36%	64%
16468	28	Coding	50 nM	66%	34%
"	"	"	100 nM	53%	47%
"	"	"	200 nM	34%	66%
"	"	"	300 nM	25%	75%
16471	28	Coding	50 nM	77%	23%
"	"	"	100 nM	56%	44%
"	"	"	200 nM	53%	47%
"	"	"	300 nM	31%	69%
14834	29	STOP	50 nM	74%	26%
"	"	"	100 nM	53%	47%
"	"	"	200 nM	24%	76%
"	"	"	300 nM	11%	89%

16473	29	STOP	50 nM	71%	29%
"	"	"	100 nM	51%	49%
"	"	"	200 nM	28%	72%
"	"	"	300 nM	23%	77%
16476	29	STOP	50 nM	74%	26%
"	"	"	100 nM	58%	42%
"	"	"	200 nM	32%	68%
"	"	"	300 nM	31%	69%

EXAMPLE 5

5 **Design and Testing of Chimeric Phosphorothioate/MMI TNF- α**
Antisense Oligodeoxynucleotides on TNF- α Levels in NeoHK Cells

10 Oligonucleotides having SEQ ID NO. 29 were synthesized as mixed phosphorothioate/methylene(methylimino) (MMI) chimeric oligodeoxynucleotides. The sequences and the oligonucleotide chemistries are shown in Table 6.

Oligonucleotide 13393 (SEQ ID NO. 49) is an antisense oligonucleotide targeted to the human intracellular adhesion molecule-1 (ICAM-1) and was used as an unrelated target control. All cytosines were 5-methyl-cytosines.

15 Dose response experiments were performed using these chimeric oligonucleotides, as discussed in Example 3 except quantitation of TNF- α mRNA levels was determined by real-time PER (RT-PER) using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PER) products in real-time. As opposed to standard PER, in which amplification products are quantitated after the
20 PER is completed, products in RT-PER are quantitated as they accumulate. This is accomplished by including in the PER reaction an oligonucleotide probe that anneals specifically between the forward and reverse PER primers, and contains two

fluorescent dyes. A reporter dye (e.g., JOE or FAM, PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PER amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular (six-second) intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

RT-PER reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PER reactions were carried out by adding 25 µl PER cocktail (1x TAQMAN7 buffer A, 5.5 mM MgCl₂, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 U RNase inhibitor, 1.25 units AMPLITAQ GOLD7, and 12.5 U MuLV reverse transcriptase) to 96 well plates containing 25 µl poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD7, 40 cycles of a two-step PER protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

For TNF- α the PER primers were:

Forward: 5'-CAGGCGGTGCTTGTTTCCT-3' SEQ ID NO. 43

Reverse: 5'-GCCAGAGGGCTGATTAGAGAGA-3' SEQ ID NO. 44 and the

PER probe was: FAM-CTTCTCCTTCCTGATCGTGGCAGGC-TAMRA (SEQ ID NO.

5 45) where FAM or JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

For GAPDH the PER primers were:

Forward primer: 5'-GAAGGTGAAGGTCGGAGTC-3' SEQ ID NO. 46

10 Reverse primer: 5'-GAAGATGGTGATGGGATTTTC-3' SEQ ID NO. 47 and the PER probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC - TAMRA 3' (SEQ ID NO. 48) where FAM or JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

15 Results are shown in Table 7. The oligonucleotide containing MMI linkages was more effective in reducing TNF- α mRNA levels than the uniformly phosphorothioate oligonucleotide. The IC₅₀ value was reduced from approximately 75 nM, for oligonucleotide 14834 (SEQ ID NO: 29), to
20 approximately 30 nM for oligonucleotide 16922 (SEQ ID NO: 29).

Dose response experiments were also performed measuring the effect on TNF- α protein levels. Protein levels were measured as described in Example 2. Results are shown in Table 8. The oligonucleotide containing four MMI linkages on
25 each end was more effective in reducing protein levels than the uniformly phosphorothioate oligonucleotide. The IC₅₀ value was reduced from approximately 90 nM, for oligonucleotide 14834 (SEQ ID NO: 29), to approximately 45 nM for oligonucleotide 16922 (SEQ ID NO: 29).

TABLE 6

**Nucleotide Sequences of Human TNF- α Chimeric
Phosphorothioate/MMI Oligodeoxynucleotides**

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
14834	GsGsAsTsGsTsTsCsGsTsCsCsTsCsCsTsCsAsCsA	29	2588-2607	STOP
16922	GmGmAmTmGsTsTsCsGsTsCsCsTsCsCsTmCmAmCmA	29	2588-2607	STOP
16923	GmGmAmTmGmTmTsCsGsTsCsCsTsCmCmTmCmAmCmA	29	2588-2607	STOP
13393	TsCsTsGsAsGsTsAsGsCsAsGsAsGsGsAsGsCsTsC	49	target control	

5

¹ All cytosine residues are 5-methyl-cytosines; "s" linkages are phosphorothioate linkages, "m" linkages are methylene(methylimino) (MMI).

10 ² Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

TABLE 7

15 **Dose Response of Chimeric Phosphorothioate/MMI TNF- α Antisense
Oligodeoxynucleotides on TNF- α mRNA Levels in PMA-Induced
NeoHK Cells**

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Express- ion	% mRNA Inhibit- ion
induced	---	---	---	100%	---
13393	49	control	25 nM	87.3%	12.7%
"	"	"	50 nM	98.5%	1.5%

"	"	"	100 nM	133.1%	---
"	"	"	200 nM	139.6%	---
14834	29	STOP	25 nM	98.7%	1.3%
"	"	"	50 nM	70.8%	29.2%
"	"	"	100 nM	36.0%	64.0%
"	"	"	200 nM	38.2%	61.8%
16922	29	STOP	25 nM	58.9%	41.1%
"	"	"	50 nM	28.2%	71.8%
"	"	"	100 nM	22.2%	77.8%
"	"	"	200 nM	18.9%	81.1%

TABLE 8

Dose Response of Chimeric Phosphorothioate/MMI TNF- α Antisense Oligodeoxynucleotides on TNF- α Protein Levels in PMA-Induced NeoHK Cells

5

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% protein Expression	% protein Inhibition
induced	---	---	---	100.0%	---
13393	49	control	25 nM	117.0%	---
"	"	"	50 nM	86.6%	13.4%
"	"	"	100 nM	98.7%	1.3%
"	"	"	200 nM	78.0%	22.0%
14834	29	STOP	25 nM	84.8%	15.2%
"	"	"	50 nM	76.9%	23.1%
"	"	"	100 nM	44.5%	55.5%
"	"	"	200 nM	18.7%	81.3%
16922	29	STOP	25 nM	67.1%	32.9%
"	"	"	50 nM	48.6%	51.4%
"	"	"	100 nM	20.0%	80.0%
"	"	"	200 nM	7.9%	92.1%
16923	29	STOP	25 nM	79.9%	20.1%
"	"	"	50 nM	69.9%	30.1%
"	"	"	100 nM	56.0%	44.0%
"	"	"	200 nM	44.5%	55.5%

EXAMPLE 6**Additional Human TNF- α Antisense Oligonucleotide Sequences**

A second screening of human TNF- α antisense oligonucleotides was performed. Oligonucleotides were designed specifically against specific regions of the TNF- α gene. A series of oligonucleotides was designed to target introns 1 and 3, and exon 4. Sequences targeting introns 1 or 3 were synthesized as uniformly phosphorothioate oligodeoxynucleotides or mixed phosphorothioate/phosphodiester chimeric backbone oligonucleotides having variable regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and deoxynucleotides. Sequences targeting exon 4 were synthesized as mixed phosphorothioate/phosphodiester chimeric backbone oligonucleotides having variable regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and deoxynucleotides. The sequences of the chimeric oligonucleotides are shown in Table 9. Sequences of the uniformly phosphorothioate oligodeoxynucleotides are shown in Table 11. These oligonucleotides were screened at 50 nM and 200 nM for their ability to inhibit TNF- α protein secretion, essentially as described in Example 2. Results for the chimeric backbone oligonucleotides are shown in Table 10; results for the uniformly phosphorothioate oligodeoxynucleotides are shown in Table 12.

For the chimeric backbone oligonucleotides targeting introns 1 or 3, oligonucleotide 21688 (SED ID NO. 69) gave 60% inhibition or greater. For chimeric backbone oligonucleotides targeting exon 4, two-thirds of the oligonucleotides gave nearly 60% inhibition or greater (SEQ ID NOS. 88, 90, 91, 92, 93, 94, 97, and 98). See Table 10. For the uniformly phosphorothioate oligodeoxynucleotides, five of nine oligonucleotides targeting intron 3 were effective in reducing TNF- α expression by nearly 60% or greater (SEQ ID NOS. 79, 80, 81, 82, and 84). See Table 12.

Oligonucleotides having SEQ ID NO. 91 and SEQ ID NO. 98 were synthesized as a uniformly phosphorothioate oligodeoxynucleotides or mixed phosphorothioate/phosphodiester chimeric backbone oligonucleotides having variable regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and deoxynucleotides. The sequences and the oligonucleotide chemistries are shown in Table 13. All 2'-MOE cytosines and 2'-deoxy cytosines were 5-methyl-cytosines.

Dose response experiments, as discussed in Example 3, were performed using these oligonucleotides. Included in this experiment were two oligonucleotides targeting intron 1 and two oligonucleotides targeting intron 3. Results are shown in Tables 14 and 15. The oligonucleotides targeting exon 4 with variable regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and deoxynucleotides and/or uniformly phosphorothioate or mixed phosphorothioate/phosphodiester were, in general, comparable to the parent compound.

Oligonucleotides targeting introns 1 or 3 having SEQ ID NOs 66, 69 and 80 were effective in reducing TNF- α mRNA levels by greater than 80% and showed a dose response effect with an IC₅₀ approximately 110 nM. See Tables 14 and 15.

TABLE 9

Nucleotide Sequences of TNF- α Chimeric Backbone (deoxy gapped) 2'-O-methoxyethyl Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
21669	ToGoCoGoTsCsTsCsTsCsAsTsTsTsCsCoCoCoToT	50	1019-1038	intron 1
21670	ToCoCoCoAsTsCsTsCsTsCsTsCsCsCsToCoToCoT	51	1039-1058	intron 1
21671	CoAoGoCoGsCsAsCsAsTsCsTsTsTsCsAoCoCoCoA	52	1059-1078	intron 1
21672	ToCoToCoTsCsTsCsAsTsCsCsCsTsCsCoCoToAoT	53	1079-1098	intron 1

21673	CoGoToCoTsTsTsCsTsCsCsAsTsGsTsToToToToT	54	1099-1118	intron 1
21674	CoAoCoAoTsCsTsCsTsTsTsCsTsGsCsAoToCoCoC	55	1119-1138	intron 1
21675	CoToCoToCsTsTsCsCsCsCsAsTsCsTsCoToToGoC	56	1139-1158	intron 1
21676	GoToCoToCsTsCsCsAsTsCsTsTsTsCsCoToToCoT	57	1159-1178	intron 1
21677	ToToCoCoAsTsGsTsGsCsCsAsGsAsCsAoToCoCoT	58	1179-1198	intron 1
21678	AoToAoCoAsCsAsCsTsTsAsGsTsGsAsGoCoAoCoC	59	1199-1218	intron 1
21679	ToToCoAoTsTsCsAsTsTsCsAsTsTsCsAoCoToCoC	60	1219-1238	intron 1
21680	ToAoToAoTsCsTsGsCsTsTsGsTsTsCsAoToToCoA	61	1239-1258	intron 1
21681	CoToGoToCsTsCsCsAsTsAsTsCsTsTsAoToToToA	62	1259-1278	intron 1
21682	ToCoToCoTsTsCsTsCsAsCsAsCsCsCsCoAoCoAoT	63	1279-1298	intron 1
21683	CoAoCoToTsGsTsTsTsCsTsTsCsCsCsCoCoAoToC	64	1299-1318	intron 1
21684	CoToCoAoCsCsAsTsCsTsTsTsAsTsTsCoAoToAoT	65	1319-1338	intron 1
21685	AoToAoToTsTsCsCsCsGsCsTsCsTsTsToCoToGoT	66	1339-1358	intron 1
21686	CoAoToCoTsCsTsCsTsCsCsTsTsAsGsCoToGoToC	67	1359-1378	intron 1
21687	ToCoToToCsTsCsTsCsCsTsTsAsTsCsToCoCoCoC	68	1379-1398	intron 1
21688	GoToGoToGsCsCsAsGsAsCsAsCsCsCsToAoToCoT	69	1399-1418	intron 1
21689	ToCoToToTsCsCsCsTsGsAsGsTsGsTsCoToToCoT	70	1419-1438	intron 1
21690	AoCoCoToTsCsCsAsGsCsAsTsTsCsAsAoCoAoGoC	71	1439-1458	intron 1
21691	CoToCoCoAsTsTsCsAsTsCsTsGsTsGsToAoToToC	72	1459-1478	intron 1
21692	ToGoAoGoGsTsGsTsCsTsGsGsTsTsTsToCoToCoT	73	1479-1498	intron 1
21693	AoCoAoCoAsTsCsCsTsCsAsGsAsGsCsToCoToToA	74	1871-1890	intron 3
21694	CoToAoGoCsCsCsTsCsCsAsAsGsTsTsCoCoAoAoG	75	1891-1910	intron 3
21695	CoGoGoGoCsTsTsCsAsAsTsCsCsCsCsAoAoAoToC	76	1911-1930	intron 3
21696	AoAoGoToTsCsTsGsCsCsTsAsCsCsAsToCoAoGoC	77	1931-1950	intron 3
21697	GoToCoCoTsTsCsTsCsAsCsAsTsTsGsToCoToCoC	78	1951-1970	intron 3
21698	CoCoToToCsCsCsTsTsGsAsGsCsTsCsAoGoCoGoA	79	1971-1990	intron 3

21699	GoGoCoCoTsGsTsGsCsTsGsTsTsCsCsToCoCoAoC	80	1991-2010	intron 3
21700	CoGoToToCsTsGsAsGsTsAsTsCsCsCsAoCoToAoA	81	2011-2030	intron 3
21701	CoAoCoAoTsCsCsCsAsCsCsTsGsGsCsCoAoToGoA	82	2031-2050	intron 3
21702	GoToCoCoTsCsTsCsTsGsTsCsTsGsTsCoAoToCoC	83	2051-2070	intron 3
21703	CoCoAoCoCsCsCsAsCsAsTsCsCsGsGsToToCoCoT	84	2071-2090	intron 3
21704	ToCoCoToGsGsCsCsCsTsCsGsAsGsCsToCoToGoC	85	2091-2110	intron 3
21705	AoToGoToCsGsGsTsTsCsAsCsTsCsTsCoCoAoCoA	86	2111-2130	intron 3
21706	AoGoAoGoGsAsGsAsGsTsCsAsGsTsGsToGoGoCoC	87	2131-2150	intron 3
21722	GoAoToCoCsCsAsAsAsGsTsAsGsAsCsCoToGoCoC	88	2561-2580	exon 4
21723	CoAoGoAoCsTsCsGsGsCsAsAsAsGsTsCoGoAoGoA	89	2541-2560	exon 4
21724	ToAoGoToCsGsGsGsCsCsGsAsTsTsGsAoToCoToC	90	2521-2540	exon 4
21725	AoGoCoGoCsTsGsAsGsTsCsGsGsTsCsAoCoCoCoT	91	2501-2520	exon 4
21726	ToCoToCoCsAsGsCsTsGsGsAsAsGsAsCoCoCoCoT	92	2481-2500	exon 4
21727	CoCoCoAoGsAsTsAsGsAsTsGsGsGsCsToCoAoToA	93	2461-2480	exon 4
21728	CoCoAoGoGsGsCsTsTsGsGsCsCsTsCsAoGoCoCoC	94	2441-2460	exon 4
21729	CoCoToCoTsGsGsGsGsTsCsTsCsCsCsToCoToGoG	95	2421-2440	exon 4
21730	CoAoGoGoGsGsCsTsCsTsTsGsAsTsGsGoCoAoGoA	96	2401-2420	exon 4
21731	GoAoGoGoAsGsGsTsTsGsAsCsCsTsTsGoGoToCoT	97	2381-2400	exon 4
21732	GoGoToAoGsGsAsGsAsCsGsGsCsGsAsToGoCoGoG	98	2361-2380	exon 4
21733	CoToGoAoTsGsGsTsGsTsGsGsGsTsGsAoGoGoAoG	99	2341-2360	exon 4

¹ Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines and 2'-deoxycytidines are 5-methyl-cytidines; "s" linkages are phosphorothioate linkages, "o" linkages are phosphodiester linkages.

² Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

TABLE 10

Dose Response of PMA-Induced neoHK Cells to Chimeric Backbone
(deoxy gapped) 2'-O-methoxyethyl TNF- α Antisense
Oligonucleotides

5

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% protein Expression	% protein Inhibition
induced	---	---	---	100%	---
14834	29	STOP	50 nM	76%	24%
"	"	"	200 nM	16%	84%
21669	50	intron 1	50 nM	134%	---
"	"	"	200 nM	114%	---
21670	51	intron 1	50 nM	122%	---
"	"	"	200 nM	101%	---
21671	52	intron 1	50 nM	90%	10%
"	"	"	200 nM	58%	42%
21672	53	intron 1	50 nM	122%	---
"	"	"	200 nM	131%	---
21673	54	intron 1	50 nM	102%	---
"	"	"	200 nM	110%	---
21674	55	intron 1	50 nM	111%	---
"	"	"	200 nM	96%	4%
21675	56	intron 1	50 nM	114%	---
"	"	"	200 nM	99%	1%
21676	57	intron 1	50 nM	107%	---
"	"	"	200 nM	96%	4%
21677	58	intron 1	50 nM	86%	14%
"	"	"	200 nM	95%	5%
21678	59	intron 1	50 nM	106%	---
"	"	"	200 nM	107%	---
21679	60	intron 1	50 nM	75%	25%
"	"	"	200 nM	73%	27%
21680	61	intron 1	50 nM	76%	24%
"	"	"	200 nM	80%	20%

21681	62	intron 1	50 nM	79%	21%
"	"	"	200 nM	82%	18%
21682	63	intron 1	50 nM	102%	---
"	"	"	200 nM	88%	12%
21683	64	intron 1	50 nM	80%	20%
"	"	"	200 nM	66%	34%
21684	65	intron 1	50 nM	91%	9%
"	"	"	200 nM	69%	31%
21685	66	intron 1	50 nM	98%	2%
"	"	"	200 nM	90%	10%
21686	67	intron 1	50 nM	97%	3%
"	"	"	200 nM	72%	28%
21687	68	intron 1	50 nM	103%	---
"	"	"	200 nM	64%	36%
21688	69	intron 1	50 nM	87%	13%
"	"	"	200 nM	40%	60%
21689	70	intron 1	50 nM	78%	22%
"	"	"	200 nM	74%	26%
21690	71	intron 1	50 nM	84%	16%
"	"	"	200 nM	80%	20%
21691	72	intron 1	50 nM	86%	14%
"	"	"	200 nM	75%	25%
21692	73	intron 1	50 nM	85%	15%
"	"	"	200 nM	61%	39%
21693	74	intron 3	50 nM	81%	19%
"	"	"	200 nM	83%	17%
21694	75	intron 3	50 nM	99%	1%
"	"	"	200 nM	56%	44%
21695	76	intron 3	50 nM	87%	13%
"	"	"	200 nM	84%	16%
21696	77	intron 3	50 nM	103%	---
"	"	"	200 nM	86%	14%
21697	78	intron 3	50 nM	99%	1%
"	"	"	200 nM	52%	48%

21698	79	intron 3	50 nM	96%	4%
"	"	"	200 nM	47%	53%
21699	80	intron 3	50 nM	73%	27%
"	"	"	200 nM	84%	16%
21700	81	intron 3	50 nM	80%	20%
"	"	"	200 nM	53%	47%
21701	82	intron 3	50 nM	94%	6%
"	"	"	200 nM	56%	44%
21702	83	intron 3	50 nM	86%	14%
"	"	"	200 nM	97%	3%
21703	84	intron 3	50 nM	88%	12%
"	"	"	200 nM	74%	26%
21704	85	intron 3	50 nM	69%	31%
"	"	"	200 nM	65%	35%
21705	86	intron 3	50 nM	92%	8%
"	"	"	200 nM	77%	23%
21706	87	intron 3	50 nM	95%	5%
"	"	"	200 nM	82%	18%
21722	88	exon 4	50 nM	81%	19%
"	"	"	200 nM	41%	59%
21723	89	exon 4	50 nM	87%	13%
"	"	"	200 nM	74%	26%
21724	90	exon 4	50 nM	68%	32%
"	"	"	200 nM	33%	67%
21725	91	exon 4	50 nM	55%	45%
"	"	"	200 nM	30%	70%
21726	92	exon 4	50 nM	72%	28%
"	"	"	200 nM	40%	60%
21727	93	exon 4	50 nM	67%	33%
"	"	"	200 nM	40%	60%
21728	94	exon 4	50 nM	62%	38%
"	"	"	200 nM	41%	59%
21729	95	exon 4	50 nM	78%	22%
"	"	"	200 nM	53%	47%

21730	96	exon 4	50 nM	68%	32%
"	"	"	200 nM	48%	52%
21731	97	exon 4	50 nM	77%	23%
"	"	"	200 nM	41%	59%
21732	98	exon 4	50 nM	62%	38%
"	"	"	200 nM	28%	72%
21733	99	exon 4	50 nM	92%	8%
"	"	"	200 nM	74%	26%

TABLE 11

**Nucleotide Sequences of Additional Human TNF- α
Phosphorothioate Oligodeoxynucleotides**

5

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
21804	TGCGTCTCTCATTTCCCCTT	50	1019-1038	intron 1
21805	TCCCATCTCTCTCCCTCTCT	51	1039-1058	intron 1
21806	CAGCGCACATCTTTCACCCA	52	1059-1078	intron 1
21807	TCTCTCTCATCCCTCCCTAT	53	1079-1098	intron 1
21808	CGTCTTTCTCCATGTTTTTT	54	1099-1118	intron 1
21809	CACATCTCTTTCTGCATCCC	55	1119-1138	intron 1
21810	CTCTCTTCCCCATCTCTTGC	56	1139-1158	intron 1
21811	GTCTCTCCATCTTTCCTTCT	57	1159-1178	intron 1
21812	TTCCATGTGCCAGACATCCT	58	1179-1198	intron 1
21813	ATACACACTTAGTGAGCACC	59	1199-1218	intron 1
21814	TTCATTCAATTCATTCCTCC	60	1219-1238	intron 1
21815	TATATCTGCTTGTTTCATTCA	61	1239-1258	intron 1
21816	CTGTCTCCATATCTTATTTA	62	1259-1278	intron 1
21817	TCTCTTCTCACACCCACAT	63	1279-1298	intron 1
21818	CACTTGTTTCTTCCCCCATC	64	1299-1318	intron 1
21819	CTCACCATCTTTATTTCATAT	65	1319-1338	intron 1
21820	ATATTTCCCGCTCTTTCTGT	66	1339-1358	intron 1

21821	CATCTCTCTCCTTAGCTGTC	67	1359-1378	intron 1
21822	TCTTCTCTCCTTATCTCCCC	68	1379-1398	intron 1
21823	GTGTGCCAGACACCCCTATCT	69	1399-1418	intron 1
21824	TCTTTCCCTGAGTGTCTTCT	70	1419-1438	intron 1
21825	ACCTTCCAGCATTCAACAGC	71	1439-1458	intron 1
21826	CTCCATTCATCTGTGTATTC	72	1459-1478	intron 1
21827	TGAGGTGTCTGGTTTTCTCT	73	1479-1498	intron 1
21828	ACACATCCTCAGAGCTCTTA	74	1871-1890	intron 3
21829	CTAGCCCTCCAAGTTCCAAG	75	1891-1910	intron 3
21830	CGGGCTTCAATCCCCAAATC	76	1911-1930	intron 3
21831	AAGTTCTGCCTACCATCAGC	77	1931-1950	intron 3
21832	GTCCTTCTCACATTGTCTCC	78	1951-1970	intron 3
21833	CCTTCCCTTGAGCTCAGCGA	79	1971-1990	intron 3
21834	GGCCTGTGCTGTTCCCTCCAC	80	1991-2010	intron 3
21835	CGTTCTGAGTATCCCCTAA	81	2011-2030	intron 3
21836	CACATCCCACCTGGCCATGA	82	2031-2050	intron 3
21837	GTCCTCTCTGTCTGTCATCC	83	2051-2070	intron 3
21838	CCACCCACATCCGGTTCCT	84	2071-2090	intron 3
21839	TCCTGGCCCTCGAGCTCTGC	85	2091-2110	intron 3
21840	ATGTCGGTTCACCTCTCCACA	86	2111-2130	intron 3
21841	AGAGGAGAGTCAGTGTGGCC	87	2131-2150	intron 3

¹ All "C" residues are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

- 5 ²Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

TABLE 12

**Dose Response of PMA-Induced neoHK Cells to TNF- α
Antisense Phosphorothioate Oligodeoxynucleotides**

5

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% protein Expression	% protein Inhibition
induced	---	---	---	100%	---
14834	29	STOP	50 nM	80%	20%
"	"	"	200 nM	13%	87%
21812	58	intron 1	50 nM	110%	---
"	"	"	200 nM	193%	---
21833	79	intron 3	50 nM	88%	12%
"	"	"	200 nM	8%	92%
21834	80	intron 3	50 nM	70%	30%
"	"	"	200 nM	18%	82%
21835	81	intron 3	50 nM	106%	---
"	"	"	200 nM	42%	58%
21836	82	intron 3	50 nM	71%	29%
"	"	"	200 nM	12%	88%
21837	83	intron 3	50 nM	129%	---
"	"	"	200 nM	74%	26%
21838	84	intron 3	50 nM	85%	15%
"	"	"	200 nM	41%	59%
21839	85	intron 3	50 nM	118%	---
"	"	"	200 nM	58%	42%
21840	86	intron 3	50 nM	120%	---
"	"	"	200 nM	96%	4%
21841	87	intron 3	50 nM	117%	---
"	"	"	200 nM	78%	22%

TABLE 13

Nucleotide Sequences of TNF- α Chimeric (deoxy gapped) 2'-O-Methoxyethyl Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
21725	AoGoCoGoCsTsGsAsGsTsCsGsGsTsCsAoCoCoCoT	91	2501-2520	exon 4
25655	AsGsCsGsCsTsGsAsGsTsCsGsGsTsCsAsCsCsCsT	"	"	"
25656	AsGsCsGsCsTsGsAsGsTsCsGsGsTsCsAsCsCsCsT	"	"	"
25660	AoGoCoGsCsTsGsAsGsTsCsGsGsTsCsAsCoCoCoT	"	"	"
21732	GoGoToAoGsGsAsGsAsCsGsGsCsGsAsToGoCoGoG	98	2361-2380	exon 4
25657	GsGsTsAsGsGsAsGsAsCsGsGsCsGsAsTsGsCsGsG	"	"	"
25658	GsGsTsAsGsGsAsGsAsCsGsGsCsGsAsTsGsCsGsG	"	"	"
25661	GoGoToAsGsGsAsGsAsCsGsGsCsGsAsTsGoCoGoG	"	"	"

¹ Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines and 2'-deoxycytidines are 5-methyl-cytidines; "s" linkages are phosphorothioate linkages, "o" linkages are phosphodiester linkages.

² Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

TABLE 14

Dose Response of 20 Hour PMA-Induced neoHK Cells to TNF- α Antisense Oligonucleotides (ASOs)

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% protein Expression	% protein Inhibition
induced	---	---	---	100%	---
14834	29	STOP	75 nM	91.2%	8.8%
"	"	"	150 nM	42.0%	58.0%
"	"	"	300 nM	16.9%	83.1%
21820	66	intron 1	75 nM	79.0%	21.0%
"	"	"	150 nM	34.5%	65.5%

"	"	"	300 nM	15.6%	84.4%
21823	69	intron 1	75 nM	79.5%	20.5%
"	"	"	150 nM	31.8%	68.2%
"	"	"	300 nM	16.2%	83.8%
21725	91	exon 4	75 nM	74.8%	25.2%
"	"	"	150 nM	58.4%	41.6%
"	"	"	300 nM	45.2%	54.8%
25655	91	exon 4	75 nM	112.0%	---
"	"	"	150 nM	55.0%	45.0%
"	"	"	300 nM	39.3%	60.7%
25656	91	exon 4	75 nM	108.3%	---
"	"	"	150 nM	60.7%	39.3%
"	"	"	300 nM	42.8%	57.2%
25660	91	exon 4	75 nM	93.2%	6.8%
"	"	"	150 nM	72.8%	27.2%
"	"	"	300 nM	50.3%	49.7%

TABLE 15

5 **Dose Response of 20 Hour PMA-Induced neoHK Cells to TNF- α
Antisense Oligonucleotides (ASOs)**

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% protein Expression	% protein Inhibition
induced	---	---	---	100%	---
14834	29	STOP	75 nM	44.9%	55.1%
"	"	"	150 nM	16.3%	83.7%
"	"	"	300 nM	2.2%	97.8%
21834	80	intron 3	75 nM	102.9%	---
"	"	"	150 nM	24.5%	75.5%
"	"	"	300 nM	19.1%	80.9%
21836	82	intron 3	75 nM	70.8%	29.2%
"	"	"	150 nM	55.9%	44.1%
"	"	"	300 nM	32.7%	67.3%

21732	98	exon 4	75 nM	42.4%	57.6%
"	"	"	150 nM	34.9%	65.1%
"	"	"	300 nM	15.4%	84.6%
25657	98	exon 4	75 nM	46.7%	53.3%
"	"	"	150 nM	72.0%	28.0%
"	"	"	300 nM	50.6%	49.4%
25658	98	exon 4	75 nM	83.7%	16.3%
"	"	"	150 nM	56.6%	43.4%
"	"	"	300 nM	36.9%	63.1%
25661	98	exon 4	75 nM	54.9%	45.1%
"	"	"	150 nM	34.4%	65.6%
"	"	"	300 nM	8.6%	91.4%

EXAMPLE 7**Activity of Fully 2'-MOE Modified TNF- α Antisense****5 Oligonucleotides**

A series of antisense oligonucleotides were synthesized targeting the terminal twenty nucleotides of each exon at every exon-intron junction of the TNF- α gene. These oligonucleotides were synthesized as fully 2'-methoxyethoxy modified oligonucleotides. The oligonucleotide sequences are shown in Table 16. Oligonucleotide 12345 (SEQ ID NO. 106) is an antisense oligonucleotide targeted to the human intracellular adhesion molecule-1 (ICAM-1) and was used as an unrelated target control.

15 The oligonucleotides were screened at 50 nM and 200 nM for their ability to inhibit TNF- α mRNA levels, as described in Example 3. Results are shown in Table 17. Oligonucleotide 21794 (SEQ ID NO. 102) showed an effect at both doses, with greater than 75% inhibition at 200 nM.

TABLE 16

**Nucleotide Sequences of Human TNF- α Uniform 2'-MOE
Oligonucleotides**

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION ³
21792	AGGCACTCACCTCTTCCCTC	100	0972-0991	E1/I1
21793	CCCTGGGGAAGTGTGGGGA	101	1579-1598	I1/E2
21794	AGACACTTACTGACTGCCTG	102	1625-1644	E2/I2
21795	GAAGATGATCCTGAAGAGGA	103	1812-1831	I2/E3
21796	GAGCTCTTACCTACAACATG	104	1860-1879	E3/I3
21797	TGAGGGTTTGCTGGAGGGAG	105	2161-2180	I3/E4
12345	GATCGCGTCGGACTATGAAG	106	target control	

¹ Emboldened residues are 2'-methoxyethoxy residues, 2'-methoxyethoxy cytosine residues are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

²Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

³ Each target region is an exon-intron junction and is represented in the form, for example, I1/E2, where I, followed by a number, refers to the intron number and E, followed by a number, refers to the exon number.

TABLE 17

**Dose Response of neoHK Cells to TNF- α
Antisense 2'-MOE Oligonucleotides**

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
induced	---	---	---	100%	---
12345	106	control	50 nM	121%	---
"	"	"	200 nM	134%	---
13393	49	control	50 nM	110%	---

"	"	"	200 nM	112%	---
14834	29	STOP	50 nM	92%	8%
"	"	"	200 nM	17%	83%
21792	100	E1/I1	50 nM	105%	---
"	"	"	200 nM	148%	---
21793	101	I1/E2	50 nM	106%	---
"	"	"	200 nM	172%	---
21794	102	E2/I2	50 nM	75%	25%
"	"	"	200 nM	23%	77%
21795	103	I2/E3	50 nM	79%	21%
"	"	"	200 nM	125%	---
21796	104	E3/I3	50 nM	56%	44%
"	"	"	200 nM	150%	---
21797	105	I3/E4	50 nM	90%	10%
"	"	"	200 nM	128%	---

EXAMPLE 8**Mouse TNF- α Oligonucleotide Sequences**

Antisense oligonucleotides were designed to target mouse
 5 TNF- α . Target sequence data are from the TNF- α cDNA sequence
 published by Semon et al. (*Nucleic Acids Res.* **1987**, 15, 9083-
 9084); Genbank accession number Y00467, provided herein as SEQ
 ID NO: 107. Oligonucleotides were synthesized primarily as
 phosphorothioate oligodeoxynucleotides. Oligonucleotide
 10 sequences are shown in Table 18. Oligonucleotide 3082 (SEQ ID
 NO. 141) is an antisense oligodeoxynucleotide targeted to the
 human intracellular adhesion molecule-1 (ICAM-1) and was used
 as an unrelated target control. Oligonucleotide 13108 (SEQ ID
 NO. 142) is an antisense oligodeoxynucleotide targeted to the
 15 herpes simplex virus type 1 and was used as an unrelated
 target control.

P388D1, mouse macrophage cells (obtained from American
 Type Culture Collection, Manassas, VA) were cultured in RPMI

1640 medium with 15% fetal bovine serum (FBS) (Life Technologies, Rockville, MD).

At assay time, cell were at approximately 90% confluency. The cells were incubated in the presence of OPTI-
5 MEM7 medium (Life Technologies, Rockville, MD), and the oligonucleotide formulated in LIPOFECTIN7 (Life Technologies), a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl
10 phosphotidylethanolamine (DOPE) in membrane filtered water. For an initial screen, the oligonucleotide concentration was 100 nM in 3 µg/ml LIPOFECTIN7. Treatment was for four hours. After treatment, the medium was removed and the cells were further incubated in RPMI medium with 15% FBS and induced with
15 10 ng/ml LPS. mRNA was analyzed 2 hours post-induction with PMA.

Total mRNA was isolated using the TOTALLY RNA™ kit (Ambion, Austin, TX), separated on a 1% agarose gel, transferred to HYBOND™-N+ membrane (Amersham, Arlington
20 Heights, IL), a positively charged nylon membrane, and probed.

A TNF-α probe consisted of the 502 bp EcoRI-HindIII fragment from BBG 56 (R&D Systems, Minneapolis, MN), a plasmid containing mouse TNF-α cDNA. A glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe consisted of the 1.06 kb HindIII
25 fragment from pHcGAP (American Type Culture Collection, Manassas, VA), a plasmid containing human G3PDH cDNA. The fragments were purified from low-melting temperature agarose, as described in Maniatis, T., et al., *Molecular Cloning: A Laboratory Manual*, 1989 and labeled with REDIVUE™ ³²P-dCTP
30 (Amersham Pharmacia Biotech, Piscataway, NJ) and PRIME-A-GENE7 labeling kit (Promega, Madison, WI). mRNA was quantitated by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Secreted TNF-α protein levels were measured using a mouse TNF-α ELISA kit (R&D Systems, Minneapolis, MN or
35 Genzyme, Cambridge, MA).

TABLE 18

**Nucleotide Sequences of Mouse TNF- α Phosphorothioate
Oligodeoxynucleotides**

5

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
14846	GAGCTTCTGCTGGCTGGCTG	108	4351-4370	5'-UTR
14847	CCTTGCTGTCCTCGCTGAGG	109	4371-4390	5'-UTR
14848	TCATGGTGTCTTTTCTGGAG	110	4511-4530	AUG
14849	CTTTCTGTGCTCATGGTGTC	111	4521-4540	AUG
14850	GCGGATCATGCTTTCTGTGC	112	4531-4550	coding
14851	GGGAGGCCATTTGGGAACTT	113	5225-5244	junction
14852	CGAATTTTGAGAAGATGATC	114	5457-5476	junction
14853	CTCCTCCACTTGGTGGTTTG	115	5799-5818	junction
14854	CCTGAGATCTTATCCAGCCT	116	6540-6559	3'-UTR
14855	CAATTACAGTCACGGCTCCC	117	6927-6946	3'-UTR
15921	CCCTTCATTCTCAAGGCACA	118	5521-5540	junction
15922	CACCCCTCAACCCGCCCCC	119	5551-5570	intron
15923	AGAGCTCTGTCTTTTCTCAG	120	5581-5600	intron
15924	CACTGCTCTGACTCTCACGT	121	5611-5630	intron
15925	ATGAGGTCCCGGTGGCCCC	122	5651-5670	intron
15926	CACCTCTGTCTTTCCACAT	123	5681-5700	intron
15927	CTCCACATCCTGAGCCTCAG	124	5731-5750	intron
15928	ATTGAGTCAGTGTCACCCTC	125	5761-5780	intron
15929	GCTGGCTCAGCCACTCCAGC	126	5821-5840	coding
15930	TCTTTGAGATCCATGCCGTT	127	5861-5880	coding
15931	AACCCATCGGCTGGCACCAC	128	5891-5910	coding
15932	GTTTGAGCTCAGCCCCCTCA	129	6061-6080	coding
15933	CTCCTCCCAGGTATATGGGC	130	6091-6110	coding
15934	TGAGTTGGTCCCCCTTCTCC	131	6121-6140	coding

15935	CAAAGTAGACCTGCCCGGAC	132	6181-6200	coding
15936	ACACCCATTCCCTTCACAGA	133	6211-6230	STOP
15937	CATAATCCCCTTTCTAAGTT	134	6321-6340	3'-UTR
15938	CACAGAGTTGGACTCTGAGC	135	6341-6360	3'-UTR
15939	CAGCATCTTGTGTTTCTGAG	136	6381-6400	3'-UTR
15940	CACAGTCCAGGTCACTGTCC	137	6401-6420	3'-UTR
15941	TGATGGTGGTGCATGAGAGG	138	6423-6442	3'-UTR
15942	GTGAATTCGGAAGCCCAT	139	6451-6470	3'-UTR
15943	CCTGACCACTCTCCCTTTGC	140	6501-6520	3'-UTR
3082	TGCATCCCCCAGGCCACCAT	141	target control	
13108	GCCGAGGTCCATGTCGTACGC	142	target control	

¹ All "C" residues are 5-methyl-cytosines except underlined "C" residues are unmodified cytosines; all linkages are phosphorothioate linkages.

5

²Co-ordinates from Genbank Accession No. Y00467, locus name "MMTNFAB", SEQ ID NO. 107.

Results are shown in Table 19. Oligonucleotides 14853
 10 (SEQ ID NO. 115), 14854 (SEQ ID NO. 116), 14855 (SEQ ID NO. 117), 15921 (SEQ ID NO. 118), 15923 (SEQ ID NO. 120), 15924 (SEQ ID NO. 121), 15925 (SEQ ID NO. 122), 15926 (SEQ ID NO. 123), 15929 (SEQ ID NO. 126), 15930 (SEQ ID NO. 127), 15931 (SEQ ID NO. 128), 15932 (SEQ ID NO. 129), 15934 (SEQ ID NO. 131),
 15 15935 (SEQ ID NO. 132), 15936 (SEQ ID NO. 133), 15937 (SEQ ID NO. 134), 15939 (SEQ ID NO. 136), 15940 (SEQ ID NO. 137), 15942 (SEQ ID NO. 139), and 15943 (SEQ ID NO. 140) gave better than 50% inhibition. Oligonucleotides 15931 (SEQ ID NO. 128), 15932 (SEQ ID NO. 129), 15934 (SEQ ID NO. 131), and
 20 15943 (SEQ ID NO. 140) gave 75% inhibition or better.

TABLE 19

Inhibition of Mouse TNF- α mRNA expression in P388D1 Cells by
Phosphorothioate Oligodeoxynucleotides

5

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
Induced	---	---	100%	0%
3082	141	control	129%	---
13664	42	control	85%	15%
14846	108	5'-UTR	84%	16%
14847	109	5'-UTR	88%	12%
14848	110	AUG	60%	40%
14849	111	AUG	75%	25%
14850	112	coding	67%	33%
14851	113	junction	62%	38%
14852	114	junction	69%	31%
14853	115	junction	49%	51%
14854	116	3'-UTR	31%	69%
14855	117	3'-UTR	39%	61%
15921	118	junction	42%	58%
15922	119	intron	64%	36%
15923	120	intron	31%	69%
15924	121	intron	29%	71%
15925	122	intron	30%	70%
15926	123	intron	29%	71%
15928	125	intron	59%	41%
15929	126	coding	38%	62%
15930	127	coding	43%	57%
15931	128	coding	23%	77%
15932	129	coding	25%	75%

15933	130	coding	52%	48%
15934	131	coding	21%	79%
15935	132	coding	39%	61%
15936	133	STOP	35%	65%
15937	134	3'-UTR	45%	55%
15938	135	3'-UTR	76%	24%
15939	136	3'-UTR	33%	67%
15940	137	3'-UTR	38%	62%
15941	138	3'-UTR	54%	46%
15942	139	3'-UTR	42%	58%
15943	140	3'-UTR	25%	75%

EXAMPLE 9**Dose response of antisense phosphorothiaote****5 oligodeoxynucleotide effects on mouse TNF- α mRNA levels in P388D1 cells**

Four of the more active oligonucleotides from the initial screen were chosen for dose response assays. These include oligonucleotides 15924 (SEQ ID NO. 121), 15931 (SEQ ID NO. 128), 15934 (SEQ ID NO. 131) and 15943 (SEQ ID NO. 140). P388D1 cells were grown, treated and processed as described in Example 8. LIPOFECTIN7 was added at a ratio of 3 μ g/ml per 100 nM of oligonucleotide. The control included LIPOFECTIN7 at a concentration of 6 μ g/ml. Results are shown in Table 20. Each oligonucleotide tested showed a dose response effect with maximal inhibition about 70% or greater and IC₅₀ values less than 50 nM.

TABLE 20

Dose Response of LPS-Induced P388D1 Cells to TNF- α
Antisense Phosphorothioate Oligodeoxynucleotides (ASOs)

5

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
induced	---	---	---	100%	---
13108	142	control	25 nM	68%	32%
"	"	"	50 nM	71%	29%
"	"	"	100 nM	64%	36%
"	"	"	200 nM	75%	25%
15924	121	intron	25 nM	63%	37%
"	"	"	50 nM	49%	51%
"	"	"	100 nM	36%	64%
"	"	"	200 nM	31%	69%
15931	128	coding	25 nM	42%	58%
"	"	"	50 nM	30%	70%
"	"	"	100 nM	17%	83%
"	"	"	200 nM	16%	84%
15934	131	coding	25 nM	37%	63%
"	"	"	50 nM	26%	74%
"	"	"	100 nM	13%	87%
"	"	"	200 nM	13%	87%
15943	140	3'-UTR	25 nM	38%	62%
"	"	"	50 nM	38%	62%

"	"	"	100 nM	16%	84%
"	"	"	200 nM	16%	84%

EXAMPLE 10

Design and Testing of 2'-O-methoxyethyl (deoxy gapped) TNF- α Antisense Oligonucleotides on TNF- α Levels in P388D1 Cells

5 Oligonucleotides having SEQ ID NO: 128, SEQ ID NO: 131, and SEQ ID NO: 140 were synthesized as uniformly phosphorothioate oligodeoxynucleotides or mixed phosphorothioate/phosphodiester chimeric oligonucleotides having variable regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and deoxynucleotides. The sequences and the oligonucleotide chemistries are shown in Table 21. All 2'-MOE cytosines were 5-methyl-cytosines. Oligonucleotides were screened as described in Example 8. Results are shown in Table 22. All the oligonucleotides tested, except

10 oligonucleotide 16817 (SEQ ID NO. 140) showed 44% or greater inhibition of TNF- α mRNA expression. Oligonucleotides 16805 (SEQ ID NO: 131), 16813 (SEQ ID NO: 140), and 16814 (SEQ ID NO: 140) showed greater than 70% inhibition.

20

TABLE 21

Nucleotide Sequences of Mouse 2'-O-methoxyethyl (deoxy gapped) TNF- α Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
15931	AsAsCsCsCsAsTsCsGsGsCsTsGsGsCsAsCsCsAsC	128	5891-5910	coding
16797	AoAoCoCsCsAsTsCsGsGsCsTsGsGsCsAsCoCoAoC	"	5891-5910	coding
16798	AsAsCsCsCsAsTsCsGsGsCsTsGsGsCsAsCsCsAsC	"	5891-5910	coding
16799	AoAoCoCoCsAsTsCsGsGsCsTsGsGsCsAoCoCoAoC	"	5891-5910	coding
16800	AsAsCsCsCsAsTsCsGsGsCsTsGsGsCsAsCsCsAsC	"	5891-5910	coding

16801	AoAoCoCoAoToCoGsGsCsTsGsGsCsAsCsCsAsC	"	5891-5910	coding
16802	AsAsCsCsCsAsTsCsGsGsCsTsGsGsCsAsCsCsAsC	"	5891-5910	coding
16803	AsAsCsCsCsAsTsCsGsGsCs ToGoGoCoAoCoCoAoC	"	5891-5910	coding
16804	AsAsCsCsCsAsTsCsGsGsCs TsGsGsCsAsCsCsAsC	"	5891-5910	coding
15934	TsGsAsGsTsTsGsGsTsCsCsCsCsCsTsTsCsTsCsC	131	6121-6140	coding
16805	ToGoAoGsTsTsGsGsTsCsCsCsCsCsTsTsCoToCoC	"	6121-6140	coding
16806	TsGsAsGsTsTsGsGsTsCsCsCsCsCsTsTsCsTsCsC	"	6121-6140	coding
16807	ToGoAoGoTsTsGsGsTsCsCsCsCsCsTsToCoToCoC	"	6121-6140	coding
16808	TsGsAsGsTsTsGsGsTsCsCsCsCsCsTsTsCsTsCsC	"	6121-6140	coding
16809	ToGoAoGoToToGoGoTsCsCsCsCsCsTsTsCsTsCsC	"	6121-6140	coding
16810	TsGsAsGsTsTsGsGsTsCsCsCsCsCsTsTsCsTsCsC	"	6121-6140	coding
16811	TsGsAsGsTsTsGsGsTsCsCs CoCoCoToToCoToCoC	"	6121-6140	coding
16812	TsGsAsGsTsTsGsGsTsCsCs CsCsCsTsTsCsTsCsC	"	6121-6140	coding
15943	CsCsTsGsAsCsCsAsCsTsCsTsCsCsCsTsTsTsGsC	140	6501-6520	3'-UTR
16813	CoCoToGsAsCsCsAsCsTsCsTsCsCsCsTsToToGoC	"	6501-6520	3'-UTR
16814	CsCsTsGsAsCsCsAsCsTsCsTsCsCsCsTsTsTsGsC	"	6501-6520	3'-UTR
16815	CoCoToGoAsCsCsAsCsTsCsTsCsCsCsToToToGoC	"	6501-6520	3'-UTR
16816	CsCsTsGsAsCsCsAsCsTsCsTsCsCsCsTsTsTsGsC	"	6501-6520	3'-UTR
16817	CoCoToGoAoCoCoAoCsTsCsTsCsCsCsTsTsTsGsC	"	6501-6520	3'-UTR
16818	CsCsTsGsAsCsCsAsCsTsCsTsCsCsCsTsTsTsGsC	"	6501-6520	3'-UTR
16819	CsCsTsGsAsCsCsAsCsTsCs ToCoCoCoToToToGoC	"	6501-6520	3'-UTR
16820	CsCsTsGsAsCsCsAsCsTsCs TsCsCsCsTsTsTsGsC	"	6501-6520	3'-UTR

¹ Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines are 5-methylcytidines; "s" linkages are phosphorothioate linkages, "o" linkages are phosphodiester linkages, "o" linkages are phosphodiester linkages.

²Co-ordinates from Genbank Accession No. Y00467, locus name "MMTNFAB", SEQ ID NO. 107.

TABLE 22

5

Inhibition of mouse TNF- α mRNA expression in P388D1 Cells by
2'-O-methoxyethyl (deoxy gapped) Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
induced	---	---	100%	0%
13108	142	control	87%	13%
15934	131	coding	28%	72%
16797	128	coding	33%	67%
16798	"	coding	34%	66%
16799	"	coding	56%	44%
16800	"	coding	35%	65%
16801	"	coding	34%	66%
16802	"	coding	38%	62%
16803	"	coding	35%	65%
16804	"	coding	39%	61%
16805	131	coding	29%	71%
16806	"	coding	31%	69%
16807	"	coding	46%	54%
16808	"	coding	43%	57%
16809	"	coding	33%	67%
16810	"	coding	37%	63%

16811	"	coding	40%	60%
16812	"	coding	31%	69%
16813	140	3'-UTR	28%	72%
16814	"	3'-UTR	28%	72%
16815	"	3'-UTR	46%	54%
16816	"	3'-UTR	49%	51%
16817	"	3'-UTR	172%	---
16818	"	3'-UTR	34%	66%
16819	"	3'-UTR	51%	49%
16820	"	3'-UTR	44%	56%

EXAMPLE 11**Effect of TNF- α Antisense Oligonucleotides in a Murine Model for Non-Insulin-dependent Diabetes Mellitus**

5 The db/db mouse model, a standard model for non-insulin-dependent diabetes mellitus (NIDDM; Hotamisligil, G.S., et al., Science, **1993**, 259, 87-90), was used to assess the activity of TNF- α antisense oligonucleotides on blood glucose levels and TNF- α mRNA levels in whole mice. These mice have elevated

10 blood glucose levels and TNF- α mRNA levels compared to wild type mice. Female db/db mice and wild-type littermates were purchased from Jackson Laboratories (Bar Harbor, ME). The effect on oligonucleotide 15931 (SEQ ID NO. 128) on blood glucose levels was determined. For determination of TNF- α

15 mRNA levels, oligonucleotide 15931 (SEQ ID NO. 128), a uniformly modified phosphorothioate oligodeoxynucleotide, was compared to oligonucleotide 25302 (SEQ ID NO. 128), a mixed phosphorothioate/phosphodiester chimeric oligonucleotide having regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and

20 deoxynucleotides. The sequences and chemistries are shown in

Table 23. Oligonucleotide 18154 (SEQ ID NO. 143) is an antisense mixed phosphorothioate/phosphodiester chimeric oligonucleotide, having regions of 2'-O-methoxyethyl (2'-MOE) nucleotides and deoxynucleotides, targeted to the human vascular cell adhesion molecule-1 (VCAM-1) and was used as an unrelated target control.

TABLE 23

Nucleotide Sequence of TNF- α Antisense Oligonucleotide

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
15931	AACCCATCGGCTGGCACCAC	128	5891-5910	coding
25302	AACCCATCGGCTGGCACCAC	128	5891-5910	coding
18154	TCAAGCAGTGCCACCGATCC	143	target control	

10

¹ All 2'-methoxyethyl cytosines and 2'-deoxy cytosines residues are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

15 ² Co-ordinates from Genbank Accession No. Y00467, locus name "MMTNFAB", SEQ ID NO. 107.

db/db mice, six to ten weeks old, were dosed intraperitoneally with oligonucleotide every other day for 2 weeks at 10 mg/kg. The mice were fasted for seven hours prior to administration of the oligonucleotide. The mice were bled via retro orbital sinus every other day, and glucose measurements were performed on the blood. Results are shown in Table 24. Oligonucleotide 15931 (SEQ ID NO. 128) was able to reduce blood glucose levels in db/db mice to levels comparable with wild type mice. Food intake between wild type mice, treated and untreated, did not differ. Food intake between db/db mice, treated and untreated, although higher than wild type mice, did not differ significantly.

30

Samples of the fat (adipose) tissue from the inguinal fat pads were taken for RNA extraction. RNA was extracted according to *Current Protocols in Molecular Biology, 1997*, Ausubel, F., et al. ed., John Wiley & Sons. RNA was purified using the RNA clean up procedure of the RNEASY7 Mini kit (Qiagen, Valencia, CA). TNF- α mRNA levels were measured using the RIBOQUANT7 kit (PharMingen, San Diego, CA) with 15 μ g of RNA per lane. The probe used was from the mCK-3b Multi-Probe Template set (PharMingen, San Diego, CA) labeled with [α^{32} P]UTP (Amersham Pharmacia Biotech, Piscataway, NJ). Results are shown in Table 25. Both oligonucleotide 15931 (SEQ ID NO. 128) and 25302 (SEQ ID NO. 128) were able to reduce TNF- α levels in fat, with 25302 (SEQ ID NO. 128) reducing TNF- α to nearly wild-type levels.

TABLE 24

Level of Blood Glucose in Normal and db/db Mice After Treatment with TNF- α Antisense Oligonucleotides

Mouse Strain	ISIS #	SEQ ID NO:	ASO Gene Target	Time (days)	blood glucose (mg/dL)
wild type	---	---	---	1	140
"	15931	128	coding	"	138
db/db	---	---	---	1	260
"	15931	128	coding	"	254
wild type	---	---	---	9	175
"	15931	128	coding	"	163
db/db	---	---	---	9	252
"	15931	128	coding	"	128

TABLE 25

Level of TNF- α mRNA in Fat of db/db Mice After Treatment with
TNF- α Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION
wt saline	---	---	100%
db/db saline	---	---	362%
18154	142	control	130%
15931	128	coding	210%
25302	128	coding	417%

5

EXAMPLE 12**Effect of TNF- α Antisense Oligonucleotides in a Murine Model
for Rheumatoid Arthritis**

Collagen-induced arthritis (CIA) was used as a murine
10 model for arthritis (Mussener, A., et al., Clin. Exp. Immunol.,
1997, 107, 485-493). Female DBA/1LacJ mice (Jackson
Laboratories, Bar Harbor, ME) between the ages of 6 and 9
weeks were used to assess the activity of TNF- α antisense
oligonucleotides. In all studies, 10 mice were used per
15 treatment group.

On day 0, the mice were immunized at the base of the
tail with 100 μ g of bovine type II collagen which was
emulsified in Complete Freund's Adjuvant (CFA). On day 7, a
second booster dose of collagen was administered by the same
20 route. On day 14, the mice were injected subcutaneously with
100 μ g of LPS. Oligonucleotide was administered
intraperitoneally (bolus) three times per week, starting on
day 0, for the duration of the 7 week study at the indicated

doses. The anti-TNF- α mAb (MM350D, Endogen, Woburn, MA) was administered intraperitoneally at 2 mg/kg once per week, starting on day 0. This antibody was formulated free of preservatives and carrier, and had an endotoxin level of 9.06 EU/mg.

Weights were recorded weekly. Mice were inspected daily for the onset of CIA, characterized by erythema and edema. Upon the onset of the disease, an assessment chart for each animal was started. Paw widths are rear ankle widths of affected and unaffected joints were measured three times a week using a constant tension caliper. Limbs were clinically evaluated and graded on a scale from 0-4, where 0=normal, 1=one digit swollen, 2=inflammation present in more than one digit, 3=joint distortion with or without inflammation, and 4=ankylosis as detected by joint manipulation. The progression of all measurements recorded to day 50. On day 50, animals were euthanized by cervical dislocation. All paws were removed and fixed in 10% neutral buffered formalin, from which histopathology slides were prepared.

Arthritis was classified into four stages based on histological evaluation of the degrees of inflammation, cartilage damage, pannus formation, bone erosion, osteolysis, fibrosis and ankylosis. Stage I is described by inflammatory cell infiltration in the tissues surrounding the joint and/or superficial layers of the synovium. Stage II is described by pannus formation with damage to the superficial layers of the cartilage. Stage III is described by subchondral bone erosion with some degree of osteolysis. Stage IV is described by severe destruction of cartilage and bone with areas of fibrosis and/or bony ankylosis. The clinical data was analyzed for differences in the incidence of disease, the onset of disease and the severity of the disease. Descriptive statistics and an analysis of variance (ANOVA) were performed. If a statistically significant difference was detected, a Dunnett's test was performed.

Two independent studies, which differed in dose range, showed that mice treated with ISIS 25302 had a reduced incidence of arthritis (Figures 1A-1B). The two dose ranges were 0.03 to 3.0 mg/kg (low range, Fig. 1A), and 2.5 to 20 mg/kg (high range, Fig. 1B). The lowest incidence of disease was observed in mice treated at doses of 3.0 (22%) and 2.5 mg/kg (38%) of ISIS 25302 respectively, as compared to the vehicle control incidence of 88% in both studies. No further reduction in the incidence of disease occurred in mice treated at higher doses. The onset of disease was delayed in groups treated with ISIS 25302, but varied between experiments (Table 1). The severity of the disease and the percent affected paws were also reduced by treatment with ISIS 25302. Best effects on these clinical outcomes were observed at 3.0 mg/kg in the low dose range study, and 2.5 and 20 mg/kg in the high dose range study.

Treatment of mice with the eight mismatch control, ISIS 30782 (5'CACCAAGCTGCGGTCCCAA 3'; SEQ ID NO: 502), yielded variable results between the low dose (Table 26A) and high dose (Table 26B) range studies. In the low dose range study, the one group treated with the control oligonucleotide, at a dose of 3.0 mg/kg, showed comparable improvements in the clinical outcome in comparison to the group treated with the anti-TNF- α oligonucleotide of equivalent dose. In contrast, the eight mismatch control oligonucleotide had minimal effects on the clinical outcome in the high dose range study, at doses of 2.5, 5.0, and 10 mg/kg; but did show effects in the clinic at the highest dose of 20 mg/kg.

TABLE 26A

Treatment	Schedule	Dose (mg/kg)	% incidence	Day of onset	Severity ("SEM)	% affected paws
Vehicle	3x/wk	-	88	18.1"0.7	7.1"2.1	59

ISIS 25302	3x/wk	0.03	70	18.6"1.1	3.1"1.2	28
ISIS 25302	3x/wk	0.1	70	17.6"0.2	3.5"1.5	30
ISIS 25302	3x/wk	0.3	44	21.5"4.5	2.9"1.4	25
ISIS 25302	3x/wk	1.0	67	21.0"3.6	3.4"1.0	36
ISIS 25302	3x/wk	3.0	22	21.5"3.5	1.2"0.8	14
TNF mAb	1x/wk	2.0	30	28.0"1.5	1.3"0.7	8.3
8MM ctrl	3x/wk	3.0	22	17.5"0.5	1.0"0.7	8.3

TABLE 26B

Treatment	Schedule	Dose (mg/kg)	% incidence	Day of Onset	Severity ("SEM)	% affected paws
Vehicle	3x/wk	-	88	17.6"0.4	6.0"1.6	53
ISIS 25302	3x/wk	2.5	38	28.3"10.8	2.1"1.5	19
ISIS 25302	3x/wk	5.0	50	23.2"5.7	4.5"1.7	40
ISIS 25302	3x/wk	10	44	17.0"0.4	4.0"1.7	33
ISIS 25302	3x/wk	20	56	23.8"5.1	2.2"1.4	19
8MM ctrl	3x/wk	2.5	71	17.4"0.7	6.3"2.2	57
8MM ctrl	3x/wk	5.0	86	20.7"3.1	6.6"2.1	57
8MM ctrl	3x/wk	10	80	18.0"0.6	6.4"1.5	55
8MM ctrl	3x/wk	20	44	19.5"1.6	1.7"1.3	17

5 In both tables, the incidence is the number of mice with
at least one affected paw/total number of mice per group.
Severity is the total clinical score/total number of mice in
the group. Percent affected paws=(number of affected paws at
10 termination/total number of paws in group) x 100. 8MM
ctrl=eight mismatch control (ISIS 30782).

Efficacy of ISIS 25302 (3 mg/kg, three times per week)
was found to be comparable to that of an anti-TNF- α mAb (2
mg/kg, once per week) as described in Table 26A. The disease
incidence in mice treated with ISIS 25302 was 22% versus 30%
15 for the group treated with the anti-TNF- α mAb. Disease

severity and percent affected paws were also reduced to a similar degree in the 3 mg/kg ISIS 25302 and anti-TNF- α mAb treated groups.

Mice treated with the anti-mTNF- α oligonucleotide, ISIS 25302, showed an improvement in the disease outcome when treated three times per week starting on the initial day of collagen-induction. Reduction of symptoms by the ISIS 25302 was dose dependent, and showed equivalent effects when compared to mice treated with an anti-TNF- α monoclonal antibody once per week from the time of collagen-induction. Histological evaluation of the joints showed a reduction in the incidence and severity of arthritic lesions in mice treated with ISIS 25302, but to a lesser extent than those mice treated with the anti-TNF- α mAb.

The efficacy of ISIS 25302 compares favorably to other anti-TNF biological agents which have been evaluated in the "classical" CIA model. For instance, treatment of mice with the recombinant human TNF receptor FC fusion protein prior to onset of disease resulted in a 28% incidence of disease as compared to 86% incidence in the saline control treated animals (Wooley, *J. Immunol.* **151**:6602-6607, 1993). In addition, preventative treatment by an anti-TNF- α antibody in the "classical" model showed 40% reduction in paw swelling in the clinic, as well as reduction in histopathological severity (Williams, *Proc. Natl. Acad. Sci. U.S.A.* **89**:9784-9788, 1992).

A marked difference was observed between the two independent studies of ISIS 25302 in this model of CIA, with respect to responsiveness of the animals to oligonucleotide treatment. Mice were more responsive to oligonucleotide treatment in the low dose range study. This responsiveness was reflected in the histological results, where all oligonucleotide treated groups showed a notable reduction in paw incidence in comparison to the vehicle group. In comparison to the high dose study, mice in the low dose study

overall displayed a lower percentage of paws with arthritic changes at the histological level.

In conclusion, evaluation of ISIS 25302 in the accelerated CIA model has shown that an anti-TNF- α

5 oligonucleotide provides an alternative approach to treatment of related human disease indications. Potential advantages of the antisense oligonucleotide therapeutic approach, over the current anti-arthritic (biological) agents, include ease of administration and a lower potential for adverse effects from
10 long term usage.

EXAMPLE 13

Effect of TNF- α Antisense Oligonucleotides in a Murine Model for Contact Sensitivity

15 Contact sensitivity is a type of immune response resulting from contact of the surface of the skin with a sensitizing chemical. A murine model for contact sensitivity is widely used to develop therapies for chronic inflammation, autoimmune disorder, and organ transplant rejection
20 (Goebeler, M., et al., Int Arch. Allergy Appl. Immunol., 1990, 93, 294-299). One example of such a disease is atopic dermatitis. Female Balb/c mice between the ages of 8 and 12 weeks are used to assess the activity of TNF- α antisense oligonucleotides in a contact sensitivity model.

25 Balb/c mice receive injections of oligonucleotide drug in saline via i.v. injection into the tail vein. The abdomen of the mice is shaved using an Oster hair clipper. The animals are anesthetized using isoflurane, and 25 μ l of 0.2% 2,4-dinitrofluorobenzene (DNFB) in 4:1 acetone:olive oil is
30 applied to the shaved abdomen two days in a row. After five days, 10 ml of 0.2% DNFB in the same vehicle is applied to the right ear. After each exposure, the mouse is suspended in air for two minutes to allow the DNFB to absorb into the skin. 24
35 and 48 hours after application of DNFB to the ear, the ear thickness is measured using a micrometer. Inflammation

(dermatitis) is indicated by a ranked thickening of the ear. Thickness of the treated ear is compared to untreated (contralateral) ear thickness.

5 **EXAMPLE 14**

Effect of TNF- α Antisense Oligonucleotides in an IL10(-/-) Murine Model for Colitis

The effects of antisense oligonucleotide-inhibition of TNF- α was studied in the IL-10^{-/-} mouse model of colitis. IL10
10 deficient mice IL-10^{-/-} display some of the features that are observed in Crohn's disease such as discontinuous lesions throughout the gastrointestinal tract and have a cytokine profile that is characteristic of a Th1 immune response. Unlike Crohn's disease, however, IL-10^{-/-} mice show a marked
15 crypt hyperplasia and absence of fissures and fistulas. In addition, IL-10^{-/-} mice have elevated levels of TNF- α expression.

Animals were treated in a prophylactic manner with one of four doses of ISIS 25302 or ISIS. Dosing extended from two
20 weeks of age, before the development of colitis, to eight weeks of age, a time at which IL-10^{-/-} mice typically exhibit advanced stages of colitis. Colitis was assessed by histological evaluation at the conclusion of the study, and the basal and induced secretion of IFN- γ and TNF- α from colon
25 organ culture supernatants was also measured at that time.

Homozygous Interleukin-10 gene-deficient mice, generated on a 129 Sv/Ev background, and 129 Sv/Ev controls were housed under specific pathogen-free conditions. Mice were housed in micro-isolator cages with tight-fitting lids containing spun-
30 polyester fiber filters. Mice were injected every other day with either ISIS 25302 or ISIS 30782 (the 8 mismatch control) at 0.01, 0.1, 1.0, and 10 mg/kg from 2-8 weeks of age via subcutaneous injection.

Animals were sacrificed using sodium pentobarbital (160
35 mg/kg). Whole colons were harvested, cut lengthwise, and fixed

in 10% phosphate-buffered formalin, paraffin-embedded, sectioned at 4 μ m, and stained with haematoxylin and eosin for light microscopic examination. The slides were reviewed independently by a pathologist in a blinded fashion and assigned a histological score for intestinal inflammation (Table 27). The total histological score represents the numerical sum of the four scoring criteria: mucosal ulceration, epithelial hyperplasia, lamina propria mononuclear cell infiltration, and lamina propria neutrophilic infiltration.

TABLE 27

Score	Mucosal ulceration	Epithelial hyperplasia	LP mononuclear infiltration	LP neutrophil infiltrate
0	Normal	Normal	Normal	Normal
1	Surface inflammation	Mild	Slight increase	Slight increase
2	Erosions	Moderate	Marked increase	Marked increase
3	Ulcerations	Pseudopolyps		

Colonic organ cultures were prepared from IL-10 gene-deficient mice treated for six weeks. Due to the patchy nature of colitis in IL-10 gene-deficient mice, whole colons were removed, cut lengthwise, flushed with PBS, and resuspended in tissue culture plates (Falcon 3046; Becton Dickinson Labware, Lincoln Park, NJ) in RPMI-1640 medium supplemented with 10% fetal calf serum, 50 mM 2-mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 U/mL). Cultures were incubated at 37° C in 5% CO₂. After 24 hours in the absence (basal) or presence of 10 μ g/ mL LPS (*E. coli*, 0111:B4, Sigma), supernatants were harvested and stored at -70° C for analysis of cytokine levels. TNF- α and IFN- γ levels in cell supernatants were measured using ELISA kits purchased from Biosource Cytoscreen (Montreal, Quebec).

Differences between treatment groups were evaluated by analysis of variance (ANOVA). Single arm analysis was performed by the Dunnett's test (SAS Institute Inc., Cary NC).

Over the 6-week treatment period, all treatment groups of IL-10 deficient mice gained weight at a similar rate (data not shown). At 8 weeks of age, IL-10^{-/-} mice displayed a patchy distribution of transmural acute and chronic inflammation, extensive mucosal ulceration, and epithelial hyperplasia. Table 28 shows the histological scores for colon tissue from IL-10^{-/-} mice treated with saline (vehicle), ISIS 25302 or ISIS 30782 (8MM ctrl) from 2 to 8 weeks of age at the indicated doses (n=6). The "total" histological score is the summation of the scores determined for each of the four histological parameters: mucosal ulceration, epithelial hyperplasia, lamina propia (LP) mononuclear cell infiltration, and lamina propria neutrophilic infiltration. Mice receiving the 0.1 mg/kg dose of the anti-TNF- α oligonucleotide, ISIS 25302, demonstrated a marked improvement in their mucosal architecture, which was statistically significant (p < 0.05) in comparison to the Vehicle (saline) group (Figure 2). No other group showed a significant histological difference in comparison to Vehicle.

TABLE 28

Treatment	Score	Mucosal ulceration	Mucosal hyperplasia	Mononuclear infiltrate	Neutrophil infiltrate	Total
Saline	Mean	1.00	1.83	2.00	1.83	6.67
	Std. Dev.	0.89	0.41	0.00	0.41	1.21
0.01 mg/kg	Mean	0.50	1.50	1.50	1.50	5.00
ISIS 25302	Std. Dev.	0.55	0.55	0.55	0.55	0.63
0.1 mg/kg	Mean	0.50	0.83	1.33	1.00	3.67
ISIS 25302	Std. Dev.	0.55	0.41	0.52	0.63	0.52

1 mg/kg	Mean	0.67	2.00	1.67	1.67	6.00
ISIS 25302	Std.	1.21	0.89	0.52	0.52	2.61
	Dev.					
10 mg/kg	Mean	1.17	1.83	1.83	1.17	6.00
ISIS 25302	Std.	1.47	0.98	0.41	0.75	2.83
	Dev.					
0.01 mg/kg	Mean	0.83	1.83	1.33	1.67	5.67
8MM ctrl	Std.	1.17	0.75	0.52	0.52	2.58
	Dev.					
0.1 mg/kg	Mean	1.00	1.67	1.33	1.17	5.17
8MM ctrl	Std.	0.63	0.52	0.52	0.52	0.63
	Dev.					
1 mg/kg	Mean	0.67	1.67	1.33	1.33	5.00
8MM ctrl	Std.	0.52	0.52	0.52	0.52	0.63
	Dev.					
10 mg/kg	Mean	0.83	2.00	1.33	1.50	5.67
8 MM ctrl	Std.	1.17	0.63	0.52	0.55	2.25
	Dev.					

Reduction of secreted TNF- α protein levels was observed in colon tissue isolated from mice treated every other day with 0.1 mg/kg of ISIS 25302 under both basal (Figure 3A) and

5 LPS-induced (Figure 3B) conditions. IFN- γ protein secretion from the isolated colon tissue was also reduced in the 0.1 mg/kg ISIS 25302 treated group relative to the saline treated group under both culture conditions (basal, Figure 4A; LPS-induced, Figure 4B). These effects were sequence specific, as

10 the eight base mismatch oligonucleotide at the same dose of 0.1 mg/kg had no effect on basal or LPS-induced TNF- α protein secretion, or LPS-induced IFN- γ secretion.

Although treatment of IL-10^{-/-} mice with an antisense oligonucleotide targeted to TNF- α had no effect on the rate at

15 which these animals gained weight, anti-TNF- α oligonucleotide treatment did have effects on several key disease parameters. Most importantly, antisense treatment at a relatively low dose

(0.1 mg/kg) significantly reduced histological signs of colitis in the mice. This included reductions in mucosal ulceration, mucosal hyperplasia, and infiltrations of mononuclear cells and neutrophils into the lamina propria of the colon. These effects were not seen with the eight-base mismatch control oligonucleotide, ISIS 30782, which indicated that the effect was sequence specific.

The histological improvement is most likely due to specific reduction in TNF- α protein levels with antisense treatment. Both the basal and LPS-induced secretion of TNF- α from colons of mice treated with 0.1 mg/kg of ISIS 25302 were reduced, while the control oligonucleotide had no effect. A decrease in basal and induced IFN- γ levels also occurred in the mice treated with 0.1 mg/kg ISIS 25302. Interruption of the proinflammatory cytokine cascade by inhibition of TNF- α expression may have abrogated the recruitment and activation of CD4+ T cells that produce IFN- γ . TNF- α is known to activate expression of key inflammatory intermediates which promote this process, including expression of cell adhesion molecules, chemokines, and other proinflammatory cytokines (Zhang et al. "Tumor necrosis factor" in *The Cytokine Handbook*, 3rd ed., Academic Press Ltd., pp. 517-547; van Deventer, *Gut* **40**:443-448, 1997).

A biphasic response to the anti-TNF- α oligonucleotide was observed in this genetically engineered mouse model of colitis, where optimal efficacy of the anti-TNF- α oligonucleotide occurred at the mid range dose of 0.1 mg/kg. Treatment at the higher doses of 1.0 and 10 mg/kg resulted in complete loss of efficacy, as observed histologically and by cytokine expression levels. The basis of this response may lie in the undefined roles of the pro- and anti-inflammatory cytokines in the absence of IL-10; and/or the pharmacokinetics and mechanism of action of the oligonucleotide.

In conclusion, ISIS 25302 reduced TNF- α expression levels in a dose and sequence-dependent manner in the IL-10 deficient mice. Specific reduction of this proinflammatory molecule diminished the pathological features associated with the intestinal injury and inflammation which occurs in the absence of IL-10 in these mice. The results from this mouse model of colitis indicate that antisense oligonucleotides to TNF- α represent a new treatment of Crohn's disease in man.

10 **EXAMPLE 15**

Effect of TNF- α Antisense Oligonucleotides in a DSS-induced Murine Model for Colitis

The pathological features of DSS-induced colitis in mice are similar in many respects to human ulcerative colitis (UC) (Table 29). This model is characterized by ulceration, epithelial damage, mucosal or transmural inflammatory infiltrate, and lymphoid hyperplasia of the colon. These effects are attributed to inappropriate macrophage function, alterations of the lumina bacteria, and the direct toxic effects of DSS on the colonic epithelium (Okayasu, *Gastroenterol.* **98**:694-702, 1990). Both acute and chronic colitis may be studied in this model, by alteration of the DSS administration schedule (Okayasu, 1990, *supra.*; Cooper et al., *Lab. Invest.* **69**:238-249, 1993). The efficacy of an anti-TNF- α mAb has been shown in both the acute and chronic model of DSS-induced colitis (Murthy et al., *Aliment. Pharmacol. Ther.* **13**:251-260, 1999; Kojouharoff et al., *Clin. Exp. Immunol.* **107**:353-358, 1997), as well as efficacy of an antisense oligonucleotide to ICAM-1 in the acute model of DSS-induced colitis (Bennett et al., *J. Pharmacol. Exp. Ther.* **280**:988-1000, 1997).

TABLE 29

Feature	Crohn's	Ulcerative colitis	DSS-induced colitis
Location	GI tract	Colon	Colon
Depth	Transmural	Mucosal	Mucosal
Extent	Discontinuous	Continuous	Continuous
Symptoms	Non-bloody diarrhea, fistula	Bloody diarrhea, no fistula	BD, no fistula
Granuloma	Yes	No	No
Genetic	Yes	Yes	Yes
Microbial	Yes	Yes	Yes
Immunological	Yes	Yes	Yes
Inflammation	Transmural	Epithelium	Epithelium
TNF- α	Elevated	Elevated	Elevated

ISIS 25302 was evaluated for efficacy in both the acute and chronic models of DSS-induced colitis. ISIS 25302 is similar in design to the human anti-TNF- α oligonucleotide, ISIS 104838, with respect to the phosphorothioate modified backbone, methylated cytosine residues, and modification of each of the five 5' and 3' sugar residues with 2'-O-(2-methoxyethyl).

Female Swiss-Webster mice, 7 to 8 weeks of age weighing 25 to 30 grams, were obtained from Taconic or Jackson Laboratory. The animals were housed at 22°C and 12 hours of dark and light cycles. Mouse chow and water were made available *ab libitum*.

Female Swiss-webster mice (n = 2) were intravenously injected with 20 mg/kg of ISIS 13920 in saline or with saline alone on day 1, 3, and 5 of the acute DSS-induced colitis protocol as described below. ISIS 13920 is a fully modified phosphorothioate oligodeoxynucleotide, 5' TCCGTCATCGCTCCTCAGGG 3' (SEQ ID NO: 503), with 2'-O-(2-methoxyethyl) modified indicated by underline. This oligonucleotide is directed to the human ras-Ha gene. Two additional groups (n = 2) of normal mice (no DSS) were subjected to the same

oligonucleotide administration protocol. Mice were sacrificed on day 7. Colons were removed, trimmed longitudinally, fixed in 10% neutral buffered formaldehyde, and processed through paraffin. Four micron sections were cut from paraffin-
5 embedded tissues, and deparaffinized by standard histological procedures. Endogenous tissue peroxidase activity was quenched with Peroxidase Blocking Reagent (DAKO; Carpinteria, CA) for 10 min at room temperature (r.t.). Tissue was treated with proteinase K (DAKO) for 10 min at r.t. to make it
10 permeable for staining. After blocking with normal donkey serum (Jackson Laboratory; Bar Harbor, Maine), the sections were incubated for 45 min at r.t. with the 2E1-B5 anti-oligonucleotide mAb (Butler et al., *Lab. Invest.*, **77**:379-388, 1997). Sections were rinsed with PBS and then incubated with
15 peroxidase conjugated rabbit anti-mouse IgG1 (Zymed Laboratories; San Francisco, CA) diluted 1:200 for 30 min at r.t. Slides were washed thoroughly with PBS and then stained for peroxidase activity by addition of 3,3'-diamino-benzidine (DAKO) for 5 min at r.t.

20 Mice received 4% dextran sodium sulfate (MW 40,000, ICN Biomedicals, Inc., Aurora OH) in double distilled water *ad libitum* from day 0 until day 5 to induce colitis. On day 5, the 4% DSS was replaced with plain drinking water.

Mice were first weighed and randomized into groups of
25 seven or eight animals. Mice were administered oligonucleotide every other day (q2d) by i.v. or s.c. injection at the indicated doses from day -2 to day 6. The vehicle group was administered 1 mL/kg 0.9% saline (Baxter Healthcare Corporation, Deerfield, Illinois) under a similar
30 treatment protocol.

Disease activity index was calculated on day 7 based on the summation of the weight, hemoccult, and stool consistency scores (Table 30). Mice were weighed initially on day 0, and then every day beginning on day 3 until time of sacrifice. The
35 stool consistency from each mouse was evaluated daily by

visible appearance, beginning on day 3. On the day of sacrifice, day 7, stool from each mouse was evaluated for occult blood using the Hemoccult test (SmithKline Diagnostics, Inc., San Jose CA). After sacrifice, the colon was removed from the ileocecal junction to the anal verge. The entire colon was then measured and observed for gross changes in the appearance of the mucosa, the total length of the colon was noted, and sections of the colon were dissected for histopathological evaluation.

10

TABLE 30

Score	Weight loss	Stool consistency	Hemoccult
0	None	Normal	Negative
1	1-5%		
2	6-10%	Loose stool	Positive
3	11-15%		
4	>15%	Diarrhea	Gross bleeding

Mice were first weighed and randomized into groups of eight to ten animals. Chronic colitis was induced by giving the mice 4% DSS in their drinking water for two cycles. For each cycle, DSS was administered until the disease activity index (DAI) reached a score of 2.0 to 2.5 (see scoring criteria below) in at least one group, at which time the 4% DSS was replaced with plain drinking water. The first cycle of DSS administration was followed by 14 days of plain drinking water. The second cycle of DSS was followed by 8 to 9 days of plain drinking water, at which time the mice were sacrificed.

Oligonucleotide was administered subcutaneously (s.c.) for four consecutive days starting on the second day of the first cycle, and then every other day thereafter at doses of 0.25 mg/kg, 2.5 mg/kg, and 12.5 mg/kg; or 0.5 and 2.5 mg/kg. TNF- α mAb was administered s.c. one time at the beginning of each cycle for a total of two treatments at 30 μ g/mouse.

30

Chronic colitis progression was determined by daily measurement of the Disease Activity Index (DAI), consisting of weight loss, stool consistency and hemoccult scores (Cooper et al., 1993, *supra.*). Each parameter was given a score (Table 5 30) and the combined score was divided by three to obtain the disease activity index (DAI). This method has been shown to correlate with the histological measures of inflammation and crypt damage.

The damage to the crypts and extent of recovery were 10 determined by histological analysis of the proximal and distal sections of the colon based on the crypt grade and percent involvement in each section. Crypt grades were scored as Grade 0 = intact crypt; Grade 1 = loss of 1/3 crypt; Grade 2 = loss of 2/3 of crypt; Grade 3 = loss of entire crypt w/intact 15 epithelium; and Grade 4 = loss of entire crypt w/loss of epithelium (ulceration). Percent involvement was scored as 1 = 1-25%; 2 = 26-50%; 3 = 51-75%; and 4 = 76-100%. Total crypt score is the combined scores of the distal and proximal colon sections. The inflammation score is the product of the grade 20 of inflammation and the extent of involvement, where Grade 0 = normal; Grade 1 = mild; Grade 2 = moderate; Grade 3 = Severe; and Percent Involvement 1 = 1-25%; 2 = 26-50%; 3 = 51-75%; 4 = 76-100%.

Total RNA was isolated from a 1 mm full length colon 25 strip from each animal using the RNeasy Mini Kit (Qiagen, Valencia CA). Mouse TNF- α and G3PDH mRNA levels were determined by standard northern blot procedures. TNF- α probe signals were normalized to G3PDH probe signal.

Differences between treatment groups were evaluated by 30 analysis of variance (ANOVA). If a statistically significant difference was detected by ANOVA then the Dunnett's test was applied (SAS Institute Inc., Cary NC).

Previous studies have examined the distribution of the "first-generation" phosphorothioate oligodeoxynucleotides in 35 colon tissue of normal and DSS-treated mice, and demonstrated

localization of oligonucleotide in both the lamina propria and the epithelial cells of the mucosal layer (Bennett, 1997, *supra.*). In this case, differences were observed between the two groups of mice with respect to degree of tissue
5 accumulation as well as relative distribution between the lamina propria and epithelial cells. Disruption of the epithelial mucosa layer and influx of immune cells into the lamina propria in the DSS-treated mice coincided with increased accumulation of the oligonucleotide in the tissue,
10 particularly in the epithelial layer.

To obtain information on the localization of a 2'-O-(2-methoxyethyl) modified (2'-MOE) phosphorothioate oligodeoxynucleotide a similar experiment was performed using immunohistochemical staining techniques, instead of
15 autoradiographic or fluorescent techniques, to detect the oligonucleotide (Butler et al., 1997, *supra.*) in the colon tissue. Immunohistochemical staining allows for direct detection of the oligonucleotide without further labeling steps during oligonucleotide synthesis. The previously
20 identified anti-oligonucleotide monoclonal antibody, 2E1, was utilized for this purpose (Butler, 1997, *supra.*). Cumulative studies have shown that the strength of the signal obtained from histological staining of an oligonucleotide with the 2E1 antibody is dependent on the oligonucleotide sequence. In
25 this respect, the staining signal for ISIS 25302 proved to be modest. For this reason we utilized ISIS 13920, a 2'-MOE modified phosphorothioate oligodeoxynucleotide with enhanced histological staining properties, to evaluate the distribution of this type of oligonucleotide in colon tissue of normal and
30 DSS-treated mice. A similar distribution and accumulation profile was observed with the "second-generation" 2'-MOE modified phosphorothioate oligodeoxynucleotide, as had previously been observed for a rhodamine labeled "first-generation" phosphorothioate oligodeoxynucleotide (Bennett,
35 1997, *supra.*). Enhanced staining by the anti-oligonucleotide

antibody, 2E1, was observed in the colon tissue of DSS-treated mice, in comparison to the normal mice.

Mice treated with ISIS 25302 every other day at a dose of 1 mg/kg in the acute model of DSS-induced colitis showed a 44% reduction in the disease activity index (DAI) relative to the saline treated control group (1.4 ± 0.2 vs 2.6 ± 0.2 ; Fig 5A). In comparison, mice treated one time with 25 micrograms of the anti-TNF- α mAb, at the commencement of DSS-induction, showed a 57 % reduction in the DAI. In both cases, the reduction in DAI was significant ($p < 0.05$) in comparison to the saline treated group. In contrast to the other two treatments, mice treated with 50 micrograms of antibody showed no improvement in the DAI. Improvement in the DAI correlated with an increase in colon length (Fig 5B). The mean colon length of the saline treated DSS-induced mice was 57% the length of normal mice (see also Okayasu, 1990, *supra.*), whereas those of the ISIS 25302 and anti-TNF- α antibody (25 μ g) treated mice were 76% and 79% respectively. The mean colon lengths of each of the two anti-TNF- α treated groups were significantly different from both the saline treated DSS-induced mice and normal mice ($p < 0.05$).

The effect of ISIS 25302 on the development of acute colitis was dose and sequence dependent (Fig. 6A-6B). A reduction of the clinical symptoms of DSS-induced colitis, as measured by the DAI, was observed in mice treated with 0.04 (60%), 0.2 (60%), and 1 mg/kg (80%) of ISIS 25302 relative to saline treated control mice. Mice treated with the eight base mismatch control oligonucleotide, ISIS 30782, showed no reduction in the DAI in comparison to the saline treated group. The reduction in DAI in mice treated with ISIS 25302 at 0.04, 0.2, and 1.0 mg/kg was statistically significant in comparison to mice treated with the eight base mismatch control oligonucleotide at 1.0 mg/kg ($p < 0.05$). A statistically significant difference was also observed between the 1.0 mg/kg ISIS 25302 group and the saline treated group.

Treatment of the mice with ISIS 25302 at the higher dose of 5 mg/kg, yielded no improvement in the DAI; as previously observed in mice treated with 50 micrograms of the anti-TNF- α mAb (described below). A partial loss of efficacy was also
5 observed in the acute DSS-induced colitis model with the anti-ICAM-1 oligonucleotide, ISIS 3082, at a dose of 5 mg/kg (Bennett, 1997, *supra.*). In the ICAM-1 study mice were administered oligonucleotide once a day for five consecutive
10 injections. Loss of efficacy, in all applications, may have resulted from an excessive accumulation of the oligonucleotide (or antibody) in the inflamed tissue, which in turn had an adverse effect on the animals (immune) response to intestinal injury by DSS.

15 ISIS 25302 was also tested for efficacy in the chronic model of DSS-induced mouse colitis. In this model, DSS was administered a second time, fourteen days after the first period of DSS administration. Animals were treated with ISIS 25302 prior to establishment of disease, starting on Day 2 of
20 the first cycle of DSS administration. A dose-dependent reduction in the clinical signs of chronic colitis was observed in the mice treated with ISIS 25302 (Fig 7A). For example, a 49% reduction (0.88 ± 0.17) in the disease activity index (DAI) was observed in mice treated at the lowest dose of
25 0.25 mg/kg of ISIS 25302, in comparison to the saline treated control group (1.7 ± 0.3) at the end of the second cycle (Day 10, Fig 7B). A greater reduction in the DAI, 86 to 87%, was observed in mice treated at the higher doses of 2.5 and 12.5 mg/kg of ISIS 25302 (0.22 ± 0.11 and 0.27 ± 0.11 , respectively).
30 In comparison, animals treated with the anti-TNF- α mAb showed a 61% reduction in DAI (0.67 ± 0.14). At this time the reductions in DAI scores were statistically significant ($p < 0.05$) in mice treated with either the anti-TNF- α mAb or ISIS 25302, at all three doses, in comparison to the vehicle group.
35 Mice that showed an improvement in DAI also showed a reduction

in inflammatory infiltrates and crypt damage at the histological level as compared to the untreated and vehicle groups (Fig. 8A-B). For example, mice treated with ISIS 25302 at 2.5 and 12.5 mg/kg demonstrated a 43% and 52% reduction in total inflammatory infiltrates (respectively), and a 43% and 48% reduction in total crypt damage relative to vehicle (Fig 8A). The proximal region of the colon was more responsive to treatment by ISIS 25302, than the distal region (Fig 8B). However, the severity of the disease was greater in the distal region of the colon.

Although not statistically significant, a thirty percent reduction in target TNF- α mRNA levels was observed in the colon tissue of mice treated at the higher doses of 2.5 and 12.5 mg/kg ISIS 25302 (Fig. 9). The TNF- α mRNA levels in colons from mice treated at the lower dose of 0.25 mg/kg of ISIS 25302 were not reduced in comparison to the vehicle group. The reduced levels of TNF- α mRNA observed for mice treated with the two higher doses of ISIS 25302 supports the dose-dependent response observed in the clinic, as measured by the DAI.

The anti-mTNF- α oligonucleotide, ISIS 25302, showed dose and sequence-specific efficacy in both the acute and chronic indications of DSS-induced colitis. ISIS 25302 treatment was also comparable in effect to treatment with an anti-TNF mAb in both indications. The reduction in the clinical symptoms observed in DSS-induced mice treated with ISIS 25302 correlated with a reduction of inflammatory infiltrates and crypt damage. Target TNF- α mRNA levels were also reduced in colon tissue derived from DSS-induced animals treated with ISIS 25302, relative to vehicle controls. The efficacy of ISIS 25302 in both the acute and chronic models of DSS-induced mouse colitis indicates that an antisense oligonucleotide which targets TNF- α mRNA represents a novel approach for treatment of human inflammatory bowel disease.

EXAMPLE 16**Effect of TNF- α Antisense Oligonucleotides in a Murine Model for Crohn's Disease**

C3H/HeJ, SJL/JK and IL10-/- mice are used in a TNBS (2,4,5,-trinitrobenzene sulfonic acid) induced colitis model for Crohn's disease (Neurath, M.F., et al., J. Exp. Med., 1995, 182, 1281-1290). Mice between the ages of 6 weeks and 3 months are used to assess the activity of TNF- α antisense oligonucleotides.

10 C3H/HeJ, SJL/JK and IL10-/- mice are fasted overnight prior to administration of TNBS. A thin, flexible polyethylene tube is slowly inserted into the colon of the mice so that the tip rests approximately 4 cm proximal to the anus. 0.5 mg of the TNBS in 50% ethanol is slowly injected
15 from the catheter fitted onto a 1 ml syringe. Animals are held inverted in a vertical position for approximately 30 seconds. TNF- α antisense oligonucleotides are administered either at the first sign of symptoms or simultaneously with induction of disease. Animals, in most cases, are dosed every
20 day. Administration is by i.v., i.p., s.q., minipumps or intracolonic injection. Experimental tissues are collected at the end of the treatment regimen for histochemical evaluation.

EXAMPLE 17

25 **Effect of TNF- α Antisense Oligonucleotides in a Murine Model for Multiple Sclerosis**

Experimental autoimmune encephalomyelitis (EAE) is a commonly accepted murine model for multiple sclerosis (Myers, K.J., et al., J. Neuroimmunol., 1992, 41, 1-8). SJL/H,
30 PL/J, (SJLxPL/J)F1, (SJLxBalb/c)F1 and Balb/c female mice between the ages of 6 and 12 weeks are used to test the activity of TNF- α antisense oligonucleotides.

The mice are immunized in the two rear foot pads and base of the tail with an emulsion consisting of encephalitogenic

protein or peptide (according to Myers, K.J., et al., J. of Immunol., 1993, 151, 2252-2260) in Complete Freund's Adjuvant supplemented with heat killed Mycobacterium tuberculosis. Two days later, the mice receive an intravenous injection of 500 ng Bordetella pertussis toxin and additional adjuvant.

Alternatively, the disease may also be induced by the adoptive transfer of T-cells. T-cells are obtained from the draining of the lymph nodes of mice immunized with encephalitogenic protein or peptide in CFA. The T cells are grown in tissue culture for several days and then injected intravenously into naive syngeneic recipients.

Mice are monitored and scored daily on a 0-5 scale for signals of the disease, including loss of tail muscle tone, wobbly gait, and various degrees of paralysis.

EXAMPLE 18

Effect of TNF- α Antisense Oligonucleotides in a Murine Model for Pancreatitis

Swiss Webster, C57BL/56, C57BL/6 lpr and gld male mice are used in an experimental pancreatitis model (Niederau, C., et al., Gastroenterology, 1985, 88, 1192-1204). Mice between the ages of 4 and 10 weeks are used to assess the activity of TNF- α antisense oligonucleotides.

Caerulein (5-200 μ g/kg) is administered i.p. every hour for one to six hours. At varying time intervals, the mice are given i.p. injection of avertin and bled by cardiac puncture. The pancreas and spleen are evaluated for histopathology and increased levels of IL-1 β , IL-6, and TNF- α . The blood is analyzed for increased levels of serum amylase and lipase. TNF- α antisense oligonucleotides are administered by intraperitoneal injection at 4 hours pre-caerulein injections.

EXAMPLE 19**Effect of TNF- α Antisense Oligonucleotides in a Murine Model for Hepatitis**

Concanavalin A-induced hepatitis is used as a murine
5 model for hepatitis (Mizuhara, H., et al., J. Exp. Med., 1994, 179, 1529-1537). It has been shown that this type of liver injury is mediated by Fas (Seino, K., et al., Gastroenterology 1997, 113, 1315-1322). Certain types of viral hepatitis, including Hepatitis C, are also mediated by Fas (J.
10 Gastroenterology and Hepatology, 1997, 12, S223-S226). Female Balb/c and C57BL/6 mice between the ages of 6 weeks and 3 months are used to assess the activity of TNF- α antisense oligonucleotides.

Mice are intravenously injected with oligonucleotide.
15 The pretreated mice are then intravenously injected with 0.3 mg concanavalin A (Con A) to induce liver injury. Within 24 hours following Con A injection, the livers are removed from the animals and analyzed for cell death (apoptosis) by *in vitro* methods. In some experiments, blood is collected from
20 the retro-orbital vein.

EXAMPLE 20**Effect of Antisense Oligonucleotide Targeted to TNF- α on Survival in Murine Heterotopic Heart Transplant Model**

25 To determine the therapeutic effects of TNF- α antisense oligonucleotides in preventing allograft rejection, murine TNF- α -specific oligonucleotides are tested for activity in a murine vascularized heterotopic heart transplant model. Hearts from Balb/c mice are transplanted into the abdominal
30 cavity of C3H mice as primary vascularized grafts essentially as described by Isobe et al., *Circulation* 1991, 84, 1246-1255. Oligonucleotide is administered by continuous intravenous administration via a 7-day Alzet pump. The mean survival time for untreated mice is usually approximately 9-10 days.

Treatment of the mice for 7 days with TNF- α antisense oligonucleotides is expected to increase the mean survival time.

5 EXAMPLE 21

Optimization of Human TNF- α Antisense Oligonucleotide

Additional antisense oligonucleotides targeted to intron 1 of human TNF- α were designed. These are shown in Table 31.

Oligonucleotides are screened by RT-PCR as described in

10 Example 5 hereinabove.

TABLE 31

Nucleotide Sequences of Human TNF- α Intron 1 Antisense Oligonucleotides

15

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
100181	AGTGTCTTCTGTGTGCCAGA	144	1409-1428	intron 1
100201	AGTGTCTTCTGTGTGCCAGA	"	"	intron 1
100230	AGTGTCTTCTGTGTGCCAGA	"	"	intron 1
100250	AGTGTCTTCTGTGTGCCAGA	"	"	intron 1
100182	GTGTCTTCTGTGTGCCAGAC	145	1408-1427	intron 1
100202	GTGTCTTCTGTGTGCCAGAC	"	"	intron 1
100231	GTGTCTTCTGTGTGCCAGAC	"	"	intron 1
100251	GTGTCTTCTGTGTGCCAGAC	"	"	intron 1
100183	TGTCTTCTGTGTGCCAGACA	146	1407-1426	intron 1
100203	TGTCTTCTGTGTGCCAGACA	"	"	intron 1
100232	TGTCTTCTGTGTGCCAGACA	"	"	intron 1
100252	TGTCTTCTGTGTGCCAGACA	"	"	intron 1

100184	GTCTTCTGTGTGCCAGACAC	147	1406-1425	intron 1
100204	GTCTTCTGTGTGCCAGACAC	"	"	intron 1
100233	GTCTTCTGTGTGCCAGACAC	"	"	intron 1
100253	GTCTTCTGTGTGCCAGACAC	"	"	intron 1
100185	TCTTCTGTGTGCCAGACACC	148	1405-1424	intron 1
100205	TCTTCTGTGTGCCAGACACC	"	"	intron 1
100234	TCTTCTGTGTGCCAGACACC	"	"	intron 1
100254	TCTTCTGTGTGCCAGACACC	"	"	intron 1
100186	CTTCTGTGTGCCAGACACCC	149	1404-1423	intron 1
100206	CTTCTGTGTGCCAGACACCC	"	"	intron 1
100235	CTTCTGTGTGCCAGACACCC	"	"	intron 1
100255	CTTCTGTGTGCCAGACACCC	"	"	intron 1
100187	TTCTGTGTGCCAGACACCCT	150	1403-1422	intron 1
100207	TTCTGTGTGCCAGACACCCT	"	"	intron 1
100236	TTCTGTGTGCCAGACACCCT	"	"	intron 1
100256	TTCTGTGTGCCAGACACCCT	"	"	intron 1
100188	TCTGTGTGCCAGACACCCTA	151	1402-1421	intron 1
100208	TCTGTGTGCCAGACACCCTA	"	"	intron 1
100237	TCTGTGTGCCAGACACCCTA	"	"	intron 1
100257	TCTGTGTGCCAGACACCCTA	"	"	intron 1
100189	CTGTGTGCCAGACACCCTAT	152	1401-1420	intron 1
100209	CTGTGTGCCAGACACCCTAT	"	"	intron 1
100238	CTGTGTGCCAGACACCCTAT	"	"	intron 1
100258	CTGTGTGCCAGACACCCTAT	"	"	intron 1

100190	TGTGTGCCAGACACCCTATC	153	1400-1419	intron 1
100210	TGTGTGCCAGACACCCTATC	"	"	intron 1
100239	TGTGTGCCAGACACCCTATC	"	"	intron 1
100259	TGTGTGCCAGACACCCTATC	"	"	intron 1
100191	TGTGCCAGACACCCTATCTT	154	1398-1417	intron 1
100211	TGTGCCAGACACCCTATCTT	"	"	intron 1
100240	TGTGCCAGACACCCTATCTT	"	"	intron 1
100260	TGTGCCAGACACCCTATCTT	"	"	intron 1
100192	GTGCCAGACACCCTATCTTC	155	1397-1416	intron 1
100212	GTGCCAGACACCCTATCTTC	"	"	intron 1
100241	GTGCCAGACACCCTATCTTC	"	"	intron 1
100261	GTGCCAGACACCCTATCTTC	"	"	intron 1
100193	TGCCAGACACCCTATCTTCT	156	1396-1415	intron 1
100213	TGCCAGACACCCTATCTTCT	"	"	intron 1
100242	TGCCAGACACCCTATCTTCT	"	"	intron 1
100262	TGCCAGACACCCTATCTTCT	"	"	intron 1
100194	GCCAGACACCCTATCTTCTT	157	1395-1414	intron 1
100214	GCCAGACACCCTATCTTCTT	"	"	intron 1
100243	GCCAGACACCCTATCTTCTT	"	"	intron 1
100263	GCCAGACACCCTATCTTCTT	"	"	intron 1
100195	CCAGACACCCTATCTTCTTC	158	1394-1413	intron 1
100215	CCAGACACCCTATCTTCTTC	"	"	intron 1
100244	CCAGACACCCTATCTTCTTC	"	"	intron 1
100264	CCAGACACCCTATCTTCTTC	"	"	intron 1

100196	CAGACACCCTATCTTCTTCT	159	1393-1412	intron 1
100216	CAGACACCCTATCTTCTTCT	"	"	intron 1
100245	CAGACACCCTATCTTCTTCT	"	"	intron 1
100265	CAGACACCCTATCTTCTTCT	"	"	intron 1
100197	AGACACCCTATCTTCTTCTC	160	1392-1411	intron 1
100217	AGACACCCTATCTTCTTCTC	"	"	intron 1
100246	AGACACCCTATCTTCTTCTC	"	"	intron 1
100266	AGACACCCTATCTTCTTCTC	"	"	intron 1
100198	GACACCCTATCTTCTTCTCT	161	1391-1410	intron 1
100218	GACACCCTATCTTCTTCTCT	"	"	intron 1
100247	GACACCCTATCTTCTTCTCT	"	"	intron 1
100267	GACACCCTATCTTCTTCTCT	"	"	intron 1
100199	ACACCCTATCTTCTTCTCTC	162	1390-1409	intron 1
100219	ACACCCTATCTTCTTCTCTC	"	"	intron 1
100248	ACACCCTATCTTCTTCTCTC	"	"	intron 1
100268	ACACCCTATCTTCTTCTCTC	"	"	intron 1
100200	CACCCTATCTTCTTCTCTCC	163	1389-1408	intron 1
100220	CACCCTATCTTCTTCTCTCC	"	"	intron 1
100249	CACCCTATCTTCTTCTCTCC	"	"	intron 1
100269	CACCCTATCTTCTTCTCTCC	"	"	intron 1
100270	GTCTTCTGTGTGCCAGAC	164	1408-1425	intron 1
100271	TCTTCTGTGTGCCAGACA	165	1407-1424	intron 1
100272	CTTCTGTGTGCCAGACAC	166	1406-1423	intron 1
100273	TTCTGTGTGCCAGACACC	167	1405-1422	intron 1

100274	TCTGTGTGCCAGACACCC	168	1404-1421	intron 1
100275	CTGTGTGCCAGACACCCT	169	1403-1420	intron 1
100276	TGTGTGCCAGACACCCTA	170	1402-1419	intron 1
100277	GTGTGCCAGACACCCTAT	171	1401-1418	intron 1
100278	TGTGCCAGACACCCTATC	172	1400-1417	intron 1
100279	TGCCAGACACCCTATCTT	173	1398-1415	intron 1
100280	GCCAGACACCCTATCTTC	174	1397-1414	intron 1
100281	CCAGACACCCTATCTTCT	175	1396-1413	intron 1
100282	CAGACACCCTATCTTCTT	176	1395-1412	intron 1
100283	AGACACCCTATCTTCTTC	177	1394-1411	intron 1
100284	GACACCCTATCTTCTTCT	178	1393-1410	intron 1
100285	ACACCCTATCTTCTTCTC	179	1392-1409	intron 1

¹ Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethyl cytosines and 2'-deoxy cytosines residues are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

²Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

EXAMPLE 22

Design of Antisense Oligonucleotides Targeting Human TNF- α

Intron 2

Additional antisense oligonucleotides targeted to intron 2 and coding regions of human TNF- α were designed. These are shown in Table 32. Oligonucleotides are screened by RT-PCR as described in Example 5 hereinabove.

TABLE 32

Nucleotide Sequences of Human TNF- α Intron 2 Antisense
Oligonucleotides

5

ISIS No.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
100549	AGAGGTTTGGAGACACTTAC	180	1635-1654	intron 2
100566	AGAGGTTTGGAGACACTTAC	"	"	intron 2
100550	GAATTAGGAAAGAGGTTTGG	181	1645-1664	intron 2
100567	GAATTAGGAAAGAGGTTTGG	"	"	intron 2
100551	CCCAAACCCAGAATTAGGAA	182	1655-1674	intron 2
100568	CCCAAACCCAGAATTAGGAA	"	"	intron 2
100552	TACCCCCAAACCCAAACCCA	183	1665-1684	intron 2
100569	TACCCCCAAACCCAAACCCA	"	"	intron 2
100553	GTACTAACCCTACCCCCAAA	184	1675-1694	intron 2
100570	GTACTAACCCTACCCCCAAA	"	"	intron 2
100554	TTCCATACCGGTACTAACCC	185	1685-1704	intron 2
100571	TTCCATACCGGTACTAACCC	"	"	intron 2
100555	CCCCCACTGCTTCCATACCG	186	1695-1714	intron 2
100572	CCCCCACTGCTTCCATACCG	"	"	intron 2
100556	CTTTAAATTTCCCCCACTGC	187	1705-1724	intron 2
100573	CTTTAAATTTCCCCCACTGC	"	"	intron 2
100557	AAGACCAAAACTTTAAATTT	188	1715-1734	intron 2
100571	AAGACCAAAACTTTAAATTT	"	"	intron 2
100558	ATCCTCCCCCAAGACCAAAA	189	1725-1744	intron 2
100640	ATCCTCCCCCAAGACCAAAA	"	"	intron 2

100559	ACCTCCATCCATCCTCCCCC	190	1735-1754	intron 2
100641	ACCTCCATCCATCCTCCCCC	"	"	intron 2
100560	CCCTACTTTCACCTCCATCC	191	1745-1764	intron 2
100642	CCCTACTTTCACCTCCATCC	"	"	intron 2
100561	GAAAATACCCCCCTACTTTC	192	1755-1774	intron 2
100643	GAAAATACCCCCCTACTTTC	"	"	intron 2
100562	AAACTTCCTAGAAAATACCC	193	1765-1784	intron 2
100644	AAACTTCCTAGAAAATACCC	"	"	intron 2
100563	TGAGACCCCTTAAACTTCCTA	194	1775-1794	intron 2
100645	TGAGACCCCTTAAACTTCCTA	"	"	intron 2
100564	AAGAAAAAGCTGAGACCCTT	195	1785-1804	intron 2
100646	AAGAAAAAGCTGAGACCCTT	"	"	intron 2
100565	GGAGAGAGAAAAGAAAAAGC	196	1795-1814	intron 2
100647	GGAGAGAGAAAAGAAAAAGC	"	"	intron 2
100575	TGAGCCAGAAGAGGTTGAGG	197	2665-2684	coding
100576	ATTCTCTTTTGTAGCCAGAA	198	2675-2694	coding
100577	TAAGCCCCCAATTCTCTTTT	199	2685-2704	coding
100578	GTTCCGACCCTAAGCCCCCA	200	2695-2714	coding
100579	CTAAGCTTGGGTTCCGACCC	201	2705-2724	coding
100580	GCTTAAAGTTCTAAGCTTGG	202	2715-2734	coding
100581	TGGTCTTGTTGCTTAAAGTT	203	2725-2744	coding
100582	TTCGAAGTGGTGGTCTTGTT	204	2735-2754	coding
100583	AATCCCAGGTTTCGAAGTGG	205	2745-2764	coding
100584	CACATTCCTGAATCCCAGGT	206	2755-2774	coding

100585	GTGCAGGCCACACATTTCCTG	207	2765-2784	coding
100586	GCACTTCACTGTGCAGGCCA	208	2775-2794	coding
100587	GTGGTTGCCAGCACTTCACT	209	2785-2804	coding
100588	TGAATTCTTAGTGGTTGCCA	210	2795-2814	coding
100589	GGCCCCAGTTTGAATTCTTA	211	2805-2824	coding
100590	GAGTTCTGGAGGCCCCAGTT	212	2815-2834	coding
100591	AGGCCCCAGTGAGTTCTGGA	32	2825-2844	coding
100592	TCAAAGCTGTAGGCCCCAGT	214	2835-2854	coding
100593	ATGTCAGGGATCAAAGCTGT	215	2845-2864	coding
100594	CAGATTCCAGATGTCAGGGA	216	2855-2874	coding
100595	CCCTGGTCTCCAGATTCCAG	217	2865-2884	coding
100596	ACCAAAGGCTCCCTGGTCTC	218	2875-2894	coding
100597	TCTGGCCAGAACCAAAGGCT	219	2885-2904	coding
100598	CCTGCAGCATTCTGGCCAGA	220	2895-2914	coding
100599	CTTCTCAAGTCCTGCAGCAT	221	2905-2924	coding
100600	TAGGTGAGGTCTTCTCAAGT	222	2915-2934	coding
100601	TGTCAATTTCTAGGTGAGGT	223	2925-2944	coding
100602	GGTCCACTTGTGTCAATTC	224	2935-2954	coding
100603	GAAGGCCTAAGGTCCACTTG	225	2945-2964	coding
100604	CTGGAGAGAGGAAGGCCTAA	226	2955-2974	coding
100605	CTGGAAACATCTGGAGAGAG	227	2965-2984	coding
100606	TCAAGGAAGTCTGGAAACAT	228	2975-2994	coding
100607	GCTCCGTGTCTCAAGGAAGT	229	2985-3004	coding
100608	ATAAATACATTCATCTGTAA	230	3085-3104	coding

100609	GGTCTCCCAAATAAATACAT	231	3095-3114	coding
100610	AGGATACCCCGGTCTCCCAA	232	3105-3124	coding
100611	TGGGTCCCCCAGGATACCCC	35	3115-3134	coding
100612	GCTCCTACATTGGGTCCCCC	234	3125-3144	coding
100613	AGCCAAGGCAGCTCCTACAT	235	3135-3154	coding
100614	AACATGTCTGAGCCAAGGCA	236	3145-3164	coding
100615	TTTCACGGAAAACATGTCTG	237	3155-3174	coding
100616	TCAGCTCCGTTTTCACGGAA	238	3165-3184	coding
100617	AGCCTATTGTTTTCAGCTCCGT	239	3175-3194	coding
100618	ACATGGGAACAGCCTATTGT	240	3185-3204	coding
100619	ATCAAAGAAGGCACAGAGG	241	3215-3234	coding
100620	GTTTAGACAACCTTAATCAGA	242	3255-3274	coding
100621	AATCAGCATTGTTTAGACAA	243	3265-3284	coding
100622	TTGGTCACCAAATCAGCATT	244	3275-3294	coding
100623	TGAGTGACAGTTGGTCACCA	245	3285-3304	coding
100624	GGCTCAGCAATGAGTGACAG	246	3295-3314	coding
100625	ATTACAGACACAACCTCCCCT	247	3325-3344	coding
100626	TAGTAGGGCGATTACAGACA	248	3335-3354	coding
100627	CGCCACTGAATAGTAGGGCG	249	3345-3364	coding
100628	CTTTATTTCTCGCCACTGAA	250	3355-3374	coding

¹ Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethyl cytosines and 2'-deoxy cytosines residues are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

² Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

Several of these oligonucleotides were chosen for dose response studies. Cells were grown and treated as described in Example 3. Results are shown in Table 33. Each
5 oligonucleotide tested showed a dose response curve with maximum inhibition greater than 75%.

TABLE 33

10 Dose Response of PMA-Induced neoHK Cells to TNF- α
Antisense Oligonucleotides (ASOs)

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% protein Expression	% protein Inhibition
induced	---	---	---	100%	---
100235	149	intron 1	75 nM	77%	23%
"	"	"	150 nM	25%	75%
"	"	"	300 nM	6%	94%
100243	157	intron 1	75 nM	68%	32%
"	"	"	150 nM	15%	85%
"	"	"	300 nM	6%	94%
100263	157	intron 1	75 nM	79%	21%
"	"	"	150 nM	30%	70%
"	"	"	300 nM	23%	77%

15 **EXAMPLE 23: Optimization of Human TNF- α Antisense
Oligonucleotide Chemistry**

Analogues of oligonucleotides 21820 (SEQ ID NO. 66) and 21823 (SEQ ID NO. 69) were designed and synthesized to find an

optimum gap size. The sequences and chemistries are shown in Table 34.

Dose response experiments were performed as described in Example 3. Results are shown in Table 35.

5

TABLE 34

Nucleotide Sequences of TNF- α Chimeric Backbone
(deoxy gapped) Oligonucleotides

10

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
21820	ATATTTCCCGCTCTTTCTGT	66	1339-1358	intron 1
28086	ATATTTCCCGCTCTTTCTGT	"	"	"
28087	ATATTTCCCGCTCTTTCTGT	"	"	"
21823	GTGTGCCAGACACCCTATCT	69	1399-1418	intron 1
28088	GTGTGCCAGACACCCTATCT	"	"	"
28089	GTGTGCCAGACACCCTATCT	"	"	"

¹ Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines and 2'-deoxycytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

15

² Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

20

TABLE 35

Dose Response of 20 Hour PMA-Induced neoHK Cells to TNF- α
Chimeric (deoxy gapped) Antisense Oligonucleotides (ASOs)

5

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% protein Expression	% protein Inhibition
induced	---	---	---	100%	---
13393	49	control	75 nM	150.0%	---
"	"	"	150 nM	135.0%	---
"	"	"	300 nM	90.0%	10.0%
21820	66	intron 1	75 nM	65.0%	35.0%
"	"	"	150 nM	28.0%	72.0%
"	"	"	300 nM	9.7%	90.3%
28086	66	intron 1	75 nM	110.0%	---
"	"	"	150 nM	83.0%	17.0%
"	"	"	300 nM	61.0%	39.0%
28087	66	intron 1	75 nM	127.0%	---
"	"	"	150 nM	143.0%	---
"	"	"	300 nM	147.0%	---
21823	69	intron 1	75 nM	35.0%	65.0%
"	"	"	150 nM	30.0%	70.0%
"	"	"	300 nM	6.4%	93.6%
28088	69	intron 1	75 nM	56.0%	44.0%
"	"	"	150 nM	26.0%	74.0%
"	"	"	300 nM	11.0%	89.0%

28089	69	intron 1	75 nM	76.0%	24.0%
"	"	"	150 nM	53.0%	47.0%
"	"	"	300 nM	23.0%	77.0%

EXAMPLE 24

**Screening of additional TNF- α chimeric (deoxy gapped)
antisense oligonucleotides**

- 5 Additional oligonucleotides targeting the major regions of TNF- α were synthesized. Oligonucleotides were synthesized as uniformly phosphorothioate chimeric oligonucleotides having regions of five 2'-O-methoxyethyl (2'-MOE) nucleotides at the wings and a central region of ten deoxynucleotides.
- 10 Oligonucleotide sequences are shown in Table 36.
- Oligonucleotides were screened as described in Example 5. Results are shown in Table 37.

TABLE 36

15

**Nucleotide Sequence of Additional Human TNF- α Chimeric
(deoxy gapped) Antisense Oligonucleotides**

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
104649	CTGAGGGAGCGTCTGCTGGC	251	0616-0635	5'-UTR
104650	CCTTGCTGAGGGAGCGTCTG	252	0621-0640	5'-UTR
104651	CTGGTCCTCTGCTGTCCTTG	253	0636-0655	5'-UTR
104652	CCTCTGCTGTCCTTGCTGAG	254	0631-0650	5'-UTR
104653	TTCTCTCCCTCTTAGCTGGT	255	0651-0670	5'-UTR
104654	TCCCTCTTAGCTGGTCCTCT	256	0646-0665	5'-UTR

104655	TCTGAGGGTTGTTTTCAGGG	257	0686-0705	5'-UTR
104656	CTGTAGTTGCTTCTCTCCCT	258	0661-0680	5'-UTR
104657	ACCTGCCTGGCAGCTTGTC	259	0718-0737	5'-UTR
104658	GGATGTGGCGTCTGAGGGTT	260	0696-0715	5'-UTR
104659	TGTGAGAGGAAGAGAACCTG	261	0733-0752	5'-UTR
104660	GAGGAAGAGAACCTGCCTGG	262	0728-0747	5'-UTR
104661	AGCCGTGGGTCAGTATGTGA	263	0748-0767	5'-UTR
104662	TGGGTCAGTATGTGAGAGGA	264	0743-0762	5'-UTR
104663	GAGAGGGTGAAGCCGTGGGT	265	0758-0777	5'-UTR
104664	TCATGGTGTCTTTCCAGGG	266	0780-0799	AUG
104665	CTTTCAGTGCTCATGGTGTC	267	0790-0809	AUG
104666	TCATGCTTTCAGTGCTCATG	268	0795-0814	AUG
104667	ACGTCCCGGATCATGCTTTC	269	0805-0824	coding
104668	GCTCCACGTCCCGGATCATG	270	0810-0829	coding
104669	TCCTCGGCCAGCTCCACGTC	271	0820-0839	coding
104670	GCGCCTCCTCGGCCAGCTCC	272	0825-0844	coding
104671	AGGAACAAGCACCGCCTGGA	273	0874-0893	coding
104672	CAAGCACCGCCTGGAGCCCT	274	0869-0888	coding
104673	AAGGAGAAGAGGCTGAGGAA	275	0889-0908	coding
104674	GAAGAGGCTGAGGAACAAGC	276	0884-0903	coding
104675	CCTGCCACGATCAGGAAGGA	277	0904-0923	coding
104676	CACGATCAGGAAGGAGAAGA	278	0899-0918	coding
104677	AAGAGCGTGGTGGCGCCTGC	279	0919-0938	coding
104678	CGTGGTGGCGCCTGCCACGA	280	0914-0933	coding

104679	AAGTGCAGCAGGCAGAAGAG	281	0934-0953	coding
104680	CAGCAGGCAGAAGAGCGTGG	282	0929-0948	coding
104681	GATCACTCCAAAGTGCAGCA	283	0944-0963	coding
104682	GGGCCGATCACTCCAAAGTG	284	0949-0968	coding
104683	GGGCCAGAGGGCTGATTAGA	285	1606-1625	coding
104684	AGAGGGCTGATTAGAGAGAG	286	1601-1620	coding
104685	GCTACAGGCTTGTCACCTCGG	287	1839-1858	coding
104686	CTGACTGCCTGGGCCAGAGG	288	1616-1635	E2/I2 ³
104687	TACAACATGGGCTACAGGCT	289	1849-1868	coding
104688	AGCCACTGGAGCTGCCCCCTC	290	2185-2204	coding
104689	CTGGAGCTGCCCCCTCAGCTT	291	2180-2199	coding
104690	TTGGCCCCGGCGGTTTCAGCCA	292	2200-2219	coding
104691	TTGGCCAGGAGGGCATTGGC	293	2215-2234	coding
104692	CCGGCGGTTTCAGCCACTGGA	294	2195-2214	coding
104693	CTCAGCTCCACGCCATTGGC	295	2230-2249	coding
104694	CAGGAGGGCATTGGCCCCGGC	296	2210-2229	coding
104695	CTCCACGCCATTGGCCAGGA	297	2225-2244	coding
104696	ACCAGCTGGTTATCTCTCAG	298	2245-2264	coding
104697	CTGGTTATCTCTCAGCTCCA	299	2240-2259	coding
104698	CCCTCTGATGGCACCACCAG	300	2260-2279	coding
104699	TGATGGCACCACCAGCTGGT	301	2255-2274	coding
104700	TAGATGAGGTACAGGCCCTC	302	2275-2294	coding
104701	AAGAGGACCTGGGAGTAGAT	303	2290-2309	coding
104702	GAGGTACAGGCCCTCTGATG	304	2270-2289	coding

104703	CAGCCTTGGCCCTTGAAGAG	305	2305-2324	coding
104704	GACCTGGGAGTAGATGAGGT	306	2285-2304	coding
104705	TTGGCCCTTGAAGAGGACCT	307	2300-2319	coding
104706	TGGTGTGGGTGAGGAGCACA	308	2337-2356	coding
104707	CGGCGATGCGGCTGATGGTG	309	2352-2371	coding
104708	TGGGTGAGGAGCACATGGGT	310	2332-2351	coding
104709	TGGTCTGGTAGGAGACGGCG	311	2367-2386	coding
104710	ATGCGGCTGATGGTGTGGGT	312	2347-2366	coding
104711	AGAGGAGGTTGACCTTGGTC	313	2382-2401	coding
104712	TGGTAGGAGACGGCGATGCG	314	2362-2381	coding
104713	AGGTTGACCTTGGTCTGGTA	315	2377-2396	coding
104714	GGCTCTTGATGGCAGAGAGG	316	2397-2416	coding
104715	TCATACCAGGGCTTGGCCTC	317	2446-2465	coding
104716	TTGATGGCAGAGAGGAGGTT	318	2392-2411	coding
104717	CCCAGATAGATGGGCTCATA	93	2461-2480	coding
104718	CCAGGGCTTGGCCTCAGCCC	94	2441-2460	coding
104719	AGCTGGAAGACCCCTCCCAG	319	2476-2495	coding
104720	ATAGATGGGCTCATAACCAGG	320	2456-2475	coding
104721	CGGTACCCCTTCTCCAGCTG	321	2491-2510	coding
104722	GAAGACCCCTCCCAGATAGA	322	2471-2490	coding
104723	ATCTCAGCGCTGAGTCGGTC	26	2506-2525	coding
104724	ACCCTTCTCCAGCTGGAAGA	323	2486-2505	coding
104725	TAGTCGGGCCGATTGATCTC	90	2521-2540	coding
104726	AGCGCTGAGTCGGTCACCCT	91	2501-2520	coding

104727	TCGGCAAAGTCGAGATAGTC	324	2536-2554	coding
104728	GGGCCGATTGATCTCAGCGC	325	2516-2535	coding
104729	TAGACCTGCCCAGACTCGGC	326	2551-2570	coding
104730	AAAGTCGAGATAGTCGGGCC	327	2531-2550	coding
104731	GCAATGATCCCAAAGTAGAC	328	2566-2585	coding
104732	CTGCCCAGACTCGGCAAAGT	329	2546-2565	coding
104733	CGTCCTCCTCACAGGGCAAT	330	2581-2600	stop
104734	GATCCCAAAGTAGACCTGCC	88	2561-2580	coding
104735	GGAAGGTTGGATGTTCTCGTCC	331	2596-2615	3'-UTR
104736	TCCTCACAGGGCAATGATCC	332	2576-2595	stop
104737	GTTGAGGGTGTCTGAAGGAG	333	2652-2671	3'-UTR
104738	GTTGGATGTTCTCGTCCTCCTC	334	2591-2610	stop
104739	TTTGAGCCAGAAGAGGTTGA	335	2667-2686	3'-UTR
104740	GAGGCGTTTGGGAAGGTTGG	336	2606-2625	3'-UTR
104741	GCCCCCAATTCTCTTTTGA	337	2682-2701	3'-UTR
104742	GCCAGAAGAGGTTGAGGGTG	338	2662-2681	3'-UTR
104743	GGGTTCCGACCCTAAGCCCC	339	2697-2716	3'-UTR
104744	CAATTCTCTTTTTGAGCCAG	340	2677-2696	3'-UTR
104745	TAAAGTTCTAAGCTTGGGTT	341	2712-2731	3'-UTR
104746	CCGACCCTAAGCCCCCAATT	342	2692-2711	3'-UTR
104747	GGTGGTCTTGTTGCTTAAAG	343	2727-2746	3'-UTR
104748	TTCTAAGCTTGGGTTCCGAC	344	2707-2726	3'-UTR
104749	CCCAGGTTTCGAAGTGGTGG	345	2742-2761	3'-UTR
104750	TCTTGTTGCTTAAAGTTCTA	346	2722-2741	3'-UTR

104751	CACACATTCCTGAATCCCAG	347	2757-2776	3'-UTR
104752	GTTTCGAAGTGGTGGTCTTG	348	2737-2756	3'-UTR
104753	CTTCACTGTGCAGGCCACAC	349	2772-2791	3'-UTR
104754	ATTCTCTGAATCCCAGGTTTC	350	2752-2771	3'-UTR
104755	TAGTGGTTGCCAGCACTTCA	351	2787-2806	3'-UTR
104756	CCCAGTTTGAATTCTTAGTG	352	2802-2821	3'-UTR
104757	CTGTGCAGGCCACACATTCC	353	2767-2786	3'-UTR
104758	GTGAGTTCTGGAGGCCCCAG	354	2817-2836	3'-UTR
104759	GTTGCCAGCACTTCACTGTG	355	2782-2801	3'-UTR
104760	TTTGAATTCTTAGTGTTGC	356	2797-2816	3'-UTR
104761	AAGCTGTAGGCCCCAGTGAG	357	2832-2851	3'-UTR
104762	TTCTGGAGGCCCCAGTTTGA	358	2812-2831	3'-UTR
104763	AGATGTCAGGGATCAAAGCT	359	2847-2866	3'-UTR
104764	TGGTCTCCAGATTCCAGATG	360	2862-2881	3'-UTR
104765	GTAGGCCCCAGTGAGTTCTG	361	2827-2846	3'-UTR
104766	GAACCAAAGGCTCCCTGGTC	362	2877-2896	3'-UTR
104767	TCAGGGATCAAAGCTGTAGG	363	2842-2861	3'-UTR
104768	TCCAGATTCCAGATGTCAGG	364	2857-2876	3'-UTR
104769	GCAGCATTCTGGCCAGAACC	365	2892-2911	3'-UTR
104770	GTCTTCTCAAGTCCTGCAGC	366	2907-2926	3'-UTR
104771	AAAGGCTCCCTGGTCTCCAG	367	2872-2891	3'-UTR
104772	CAATTCTAGGTGAGGTCTT	368	2922-2941	3'-UTR
104773	ATTCTGGCCAGAACCAAAGG	369	2887-2906	3'-UTR
104774	CTCAAGTCCTGCAGCATTCT	34	2902-2921	3'-UTR

104775	AAGGTCCACTTGTGTCAATT	370	2937-2956	3'-UTR
104776	GAGAGAGGAAGGCCTAAGGT	371	2952-2971	3'-UTR
104777	TCTAGGTGAGGTCTTCTCAA	372	2917-2936	3'-UTR
104778	CCACTTGTGTCAATTTCTAG	373	2932-2951	3'-UTR
104779	GTCTGGAAACATCTGGAGAG	374	2967-2986	3'-UTR
104780	CCGTGTCTCAAGGAAGTCTG	375	2982-3001	3'-UTR
104781	AGGAAGGCCTAAGGTCCACT	376	2947-2966	3'-UTR
104782	GAGGGAGCTGGCTCCATGGG	377	3014-3033	3'-UTR
104783	GAAACATCTGGAGAGAGGAA	378	2962-2981	3'-UTR
104784	GTGCAAACATAAATAGAGGG	379	3029-3048	3'-UTR
104785	TCTCAAGGAAGTCTGGAAC	380	2977-2996	3'-UTR
104786	AATAAATAATCACAAGTGCA	381	3044-3063	3'-UTR
104787	GGGCTGGGCTCCGTGTCTCA	382	2992-3011	3'-UTR
104788	TACCCCGGTCTCCCAAATAA	383	3101-3120	3'-UTR
104789	AACATAAATAGAGGGAGCTG	384	3024-3043	3'-UTR
104790	TTGGGTCCCCCAGGATACCC	385	3116-3135	3'-UTR
104791	ATAATCACAAGTGCAAACAT	386	3039-3058	3'-UTR
104792	AAGGCAGCTCCTACATTGGG	387	3131-3150	3'-UTR
104793	CGGTCTCCCAAATAAATACA	388	3096-3115	3'-UTR
104794	AAACATGTCTGAGCCAAGGC	389	3146-3165	3'-UTR
104795	TCCCCCAGGATACCCCGGTC	390	3111-3130	3'-UTR
104796	AGCTCCTACATTGGGTCCCC	391	3126-3145	3'-UTR
104797	CTCCGTTTTTCACGGAAAACA	37	3161-3180	3'-UTR
104798	TGTCTGAGCCAAGGCAGCTC	392	3141-3160	3'-UTR

104799	CAGCCTATTGTTTCAGCTCCG	393	3176-3195	3'-UTR
104800	AGAAGGCACAGAGGCCAGGG	394	3209-3228	3'-UTR
104801	TTTTACGGAAAACATGTCT	395	3156-3175	3'-UTR
104802	TATTGTTTCAGCTCCGTTTTTC	396	3171-3190	3'-UTR
104803	AAAAACATAATCAAAAGAAG	397	3224-3243	3'-UTR
104804	CAGATAAATATTTTAAAAAA	398	3239-3258	3'-UTR
104805	TACATGGGAACAGCCTATTG	399	3186-3205	3'-UTR
104806	TTTAGACAACCTTAATCAGAT	400	3254-3273	3'-UTR
104807	CATAATCAAAAAGAAGGCACA	401	3219-3238	3'-UTR
104808	ACCAAATCAGCATTGTTTAG	402	3269-3288	3'-UTR
104809	AAATATTTTAAAAACATAA	403	3234-3253	3'-UTR
104810	GAGTGACAGTTGGTCACCAA	404	3284-3303	3'-UTR
104811	ACAACTTAATCAGATAAATA	405	3249-3268	3'-UTR
104812	CAGAGGCTCAGCAATGAGTG	406	3299-3318	3'-UTR
104813	ATCAGCATTGTTTAGACAAC	407	3264-3283	3'-UTR
104814	AGGGCGATTACAGACACAAC	408	3331-3350	3'-UTR
104815	ACAGTTGGTCACCAAATCAG	409	3279-3298	3'-UTR
104816	TCGCCACTGAATAGTAGGGC	410	3346-3365	3'-UTR
104817	GCTCAGCAATGAGTGACAGT	411	3294-3313	3'-UTR
104818	AGCAAACCTTTATTTCTCGCC	412	3361-3380	3'-UTR
104819	GATTACAGACACAACCTCCCC	413	3326-3345	3'-UTR
104820	ACTGAATAGTAGGGCGATTA	414	3341-3360	3'-UTR
104821	ACTTTATTTCTCGCCACTGA	415	3356-3375	3'-UTR
104822	GCTGTCCTTGCTGAGGGAGC	416	0626-0645	5'-UTR

104823	CTTAGCTGGTCCTCTGCTGT	417	0641-0660	5'-UTR
104824	GTTGCTTCTCTCCCTCTTAG	418	0656-0675	5'-UTR
104825	TGGCGTCTGAGGGTTGTTTT	419	0691-0710	5'-UTR
104826	AGAGAACCTGCCTGGCAGCT	420	0723-0742	5'-UTR
104827	CAGTATGTGAGAGGAAGAGA	421	0738-0757	5'-UTR
104828	GGTGAAGCCGTGGGTCACTA	422	0753-0772	5'-UTR
104829	AGTGCTCATGGTGTCTTTTC	423	0785-0804	AUG
104830	CCGGATCATGCTTTCACTGC	424	0800-0819	coding
104831	GGCCAGCTCCACGTCCCGGA	425	0815-0834	coding
104832	GGCCCCCTGTCTTCTTGGG	426	0847-0866	coding
104833	GGCTGAGGAACAAGCACCGC	427	0879-0898	coding
104834	TCAGGAAGGAGAAGAGGCTG	428	0894-0913	coding
104835	TGGCGCCTGCCACGATCAGG	429	0909-0918	coding
104836	GGCAGAAGAGCGTGGTGGCG	430	0924-0943	coding
104837	CTCCAAAGTGCAGCAGGCAG	431	0939-0958	coding
104838	GCTGATTAGAGAGAGGTCCC	432	1596-1615	coding
104839	TGCCTGGGCCAGAGGGCTGA	433	1611-1630	coding
104840	GCTGCCCCCTCAGCTTGAGGG	434	2175-2194	coding
104841	GGTTCAGCCACTGGAGCTGC	435	2190-2209	coding
104842	GGGCATTGGCCCGGCGGTTC	436	2205-2224	coding
104843	CGCCATTGGCCAGGAGGGCA	437	2220-2239	coding
104844	TATCTCTCAGCTCCACGCCA	438	2235-2254	coding
104845	GCACCACCAGCTGGTTATCT	439	2250-2269	coding
104846	ACAGGCCCTCTGATGGCACC	440	2265-2284	coding

104847	GGGAGTAGATGAGGTACAGG	441	2280-2299	coding
104848	CCTTGAAGAGGACCTGGGAG	442	2295-2314	coding
104849	GAGGAGCACATGGGTGGAGG	443	2327-2346	coding
104850	GCTGATGGTGTGGGTGAGGA	444	2342-2361	coding
104851	GGAGACGGCGATGCGGCTGA	445	2357-2376	coding
104852	GACCTTGGTCTGGTAGGAGA	446	2372-2391	coding
104853	GGCAGAGAGGAGGTTGACCT	447	2387-2406	coding
104854	GCTTGGCCTCAGCCCCCTCT	23	2436-2455	coding
104855	TGGGCTCATACCAGGGCTTG	448	2451-2470	coding
104856	CCCCTCCCAGATAGATGGGC	449	2466-2485	coding
104857	TCTCCAGCTGGAAGACCCCT	92	2481-2500	coding
104858	TGAGTCGGTCACCCTTCTCC	450	2496-2515	coding
104859	GATTGATCTCAGCGCTGAGT	451	2511-2530	coding
104860	CGAGATAGTCGGGCCGATTG	452	2526-2545	coding
104861	CAGACTCGGCAAAGTCGAGA	89	2541-2560	coding
104862	CAAAGTAGACCTGCCCAGAC	453	2556-2575	coding
104863	ACAGGGCAATGATCCCAAAG	454	2571-2590	stop
104864	ATGTTTCGTCCTCCTCACAGG	455	2586-2605	stop
104865	GTTTGGGAAGGTTGGATGTT	456	2601-2620	3'-UTR
104866	AAGAGGTTGAGGGTGTCTGA	457	2657-2676	3'-UTR
104867	CTCTTTTTGAGCCAGAAGAG	458	2672-2691	3'-UTR
104868	CCTAAGCCCCCAATTCTCTT	459	2687-2706	3'-UTR
104869	AGCTTGGGTTCCGACCCTAA	460	2702-2721	3'-UTR

104870	TTGCTTAAAGTTCTAAGCTT	461	2717-2736	3'-UTR
104871	GAAGTGGTGGTCTTGTTGCT	462	2732-2751	3'-UTR
104872	TGAATCCCAGGTTTCGAAGT	463	2747-2766	3'-UTR
104873	CAGGCCACACATTCCTGAAT	464	2762-2781	3'-UTR
104874	CAGCACTTCACTGTGCAGGC	465	2777-2796	3'-UTR
104875	ATTCTTAGTGGTTGCCAGCA	466	2792-2811	3'-UTR
104876	GAGGCCCCAGTTTGAATTCT	467	2807-2826	3'-UTR
104877	CCCCAGTGAGTTCTGGAGGC	468	2822-2841	3'-UTR
104878	GATCAAAGCTGTAGGCCCCA	469	2837-2856	3'-UTR
104879	ATTCCAGATGTCAGGGATCA	470	2852-2871	3'-UTR
104880	CTCCCTGGTCTCCAGATTCC	471	2867-2886	3'-UTR
104881	GGCCAGAACC AAAGGCTCCC	472	2882-2901	3'-UTR
104882	GTCCTGCAGCATTCTGGCCA	473	2897-2916	3'-UTR
104883	GTGAGGTCTTCTCAAGTCCT	474	2912-2931	3'-UTR
104884	TGTGTCAATTTCTAGGTGAG	475	2927-2946	3'-UTR
104885	GGCCTAAGGTCCACTTGTGT	476	2942-2961	3'-UTR
104886	ATCTGGAGAGAGGAAGGCCT	477	2957-2976	3'-UTR
104887	AGGAAGTCTGGAAACATCTG	478	2972-2991	3'-UTR
104888	GGGCTCCGTGTCTCAAGGAA	479	2987-3006	3'-UTR
104889	AAATAGAGGGAGCTGGCTCC	480	3019-3038	3'-UTR
104890	CACAAGTGCAAACATAAATA	481	3034-3053	3'-UTR
104891	TCCCAAATAAATACATTCAT	482	3091-3110	3'-UTR
104892	CAGGATACCCCGGTCTCCCA	483	3106-3125	3'-UTR
104893	CTACATTGGGTCCCCCAGGA	484	3121-3140	3'-UTR

104894	GAGCCAAGGCAGCTCCTACA	485	3136-3155	3'-UTR
104895	ACGGAAAACATGTCTGAGCC	486	3151-3170	3'-UTR
104896	TTCAGCTCCGTTTTTCACGGA	487	3166-3185	3'-UTR
104897	GGGAACAGCCTATTGTTTCAG	488	3181-3200	3'-UTR
104898	TCAAAAGAAGGCACAGAGGC	489	3214-3233	3'-UTR
104899	TTTTAAAAAACATAATCAAA	490	3229-3248	3'-UTR
104900	TTAATCAGATAAATATTTTA	491	3244-3263	3'-UTR
104901	CATTGTTTAGACAACCTTAAT	492	3259-3278	3'-UTR
104902	TGGTCACCAAATCAGCATTG	493	3274-3293	3'-UTR
104903	GCAATGAGTGACAGTTGGTC	494	3289-3308	3'-UTR
104904	GGGAGCAGAGGCTCAGCAAT	495	3304-3323	3'-UTR
104905	ATAGTAGGGCGATTACAGAC	496	3336-3355	3'-UTR
104906	ATTTCTCGCCACTGAATAGT	497	3351-3370	3'-UTR

¹ Emboldened residues are 2'-O-methoxyethyl residues (others are 2'-deoxy-). All 2'-O-methoxyethyl cytosines and 2'-deoxy cytosines residues are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

²Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

³ This target region is an exon-intron junction and is represented in the form, for example, I1/E2, where I, followed by a number, refers to the intron number and E, followed by a number, refers to the exon number.

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TABLE 37

Inhibition of Human TNF- α mRNA Expression by Chimeric
(deoxy gapped) Phosphorothioate Oligodeoxynucleotides

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ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
basal	---	---	0.0%	---
induced	---	---	100.0%	0.0%
28089	69	intron 1	42.3%	57.7%
104649	251	5'-UTR	165.6%	---
104650	252	5'-UTR	75.8%	24.2%
104651	253	5'-UTR	58.2%	41.8%
104652	254	5'-UTR	114.5%	---
104653	255	5'-UTR	84.9%	15.1%
104654	256	5'-UTR	80.8%	19.2%
104655	257	5'-UTR	94.3%	5.7%
104656	258	5'-UTR	78.4%	21.6%
104657	259	5'-UTR	87.4%	12.6%
104658	260	5'-UTR	213.4%	---
104659	261	5'-UTR	96.3%	3.7%
104660	262	5'-UTR	153.1%	---
104661	263	5'-UTR	90.0%	10.0%
104662	264	5'-UTR	33.3%	66.7%
104663	265	5'-UTR	144.2%	---
104664	266	AUG	76.3%	23.7%

104665	267	AUG	185.3%	---
104666	268	AUG	67.4%	32.6%
104667	269	Coding	94.3%	5.7%
104668	270	Coding	63.1%	36.9%
104669	271	Coding	50.8%	49.2%
104670	272	Coding	43.7%	56.3%
104671	273	Coding	52.2%	47.8%
104672	274	Coding	51.8%	48.2%
104673	275	Coding	102.3%	---
104674	276	Coding	135.4%	---
104675	277	Coding	83.1%	16.9%
104676	278	Coding	87.5%	12.5%
104677	279	Coding	53.6%	46.4%
104678	280	Coding	75.2%	24.8%
104679	281	Coding	114.0%	---
104680	282	Coding	142.5%	---
104681	283	Coding	58.5%	41.5%
104682	284	Coding	101.9%	---
104683	285	Coding	77.1%	22.9%
104684	286	Coding	61.0%	39.0%
104685	287	Coding	65.9%	34.1%
104686	288	E2/I2	59.2%	40.8%
104687	289	Coding	77.0%	23.0%
104688	290	Coding	40.1%	59.9%
104689	291	Coding	78.6%	21.4%

104690	292	Coding	90.9%	9.1%
104691	293	Coding	107.6%	---
104692	294	Coding	63.4%	36.6%
104693	295	Coding	74.1%	25.9%
104694	296	Coding	108.3%	---
104695	297	Coding	48.2%	51.8%
104696	298	Coding	120.3%	---
104697	299	Coding	45.0%	55.0%
104698	300	Coding	77.1%	22.9%
104699	301	Coding	143.7%	---
104700	302	Coding	96.1%	3.9%
104701	303	Coding	106.8%	---
104702	304	Coding	157.4%	---
104703	305	Coding	84.3%	15.7%
104704	306	Coding	182.8%	---
104705	307	Coding	125.1%	---
104706	308	Coding	81.8%	18.2%
104707	309	Coding	104.8%	---
104708	310	Coding	163.0%	---
104709	311	Coding	95.0%	5.0%
104710	312	Coding	182.1%	---
104711	313	Coding	82.1%	17.9%
104712	314	Coding	118.1%	---
104713	315	Coding	31.1%	68.9%
104714	316	Coding	90.5%	9.5%

104715	317	Coding	96.7%	3.3%
104716	318	Coding	180.7%	---
104717	93	Coding	71.6%	28.4%
104718	94	Coding	187.0%	---
104719	319	Coding	88.8%	11.2%
104720	320	Coding	166.5%	---
104721	321	Coding	65.0%	35.0%
104722	322	Coding	59.6%	40.4%
104723	26	Coding	90.1%	9.9%
104724	323	Coding	88.7%	11.3%
104725	90	Coding	94.7%	5.3%
104726	91	Coding	84.1%	15.9%
104727	324	Coding	125.3%	---
104728	325	Coding	221.7%	---
104729	326	Coding	102.4%	---
104730	327	Coding	151.6%	---
104731	328	Coding	102.2%	---
104732	329	Coding	53.2%	46.8%
104733	330	Stop	57.0%	43.0%
104734	88	Coding	119.2%	---
104735	331	3'-UTR	71.2%	28.8%
104736	332	Stop	79.0%	21.0%
104737	333	3'-UTR	87.4%	12.6%
104738	334	Stop	36.8%	63.2%
104739	335	3'-UTR	106.0%	---

104740	336	3'-UTR	130.9%	---
104741	337	3'-UTR	79.2%	20.8%
104742	338	3'-UTR	159.0%	---
104743	339	3'-UTR	96.1%	3.9%
104744	340	3'-UTR	129.9%	---
104745	341	3'-UTR	80.2%	19.8%
104746	342	3'-UTR	168.8%	---
104747	343	3'-UTR	89.2%	10.8%
104748	344	3'-UTR	103.4%	---
104749	345	3'-UTR	89.0%	11.0%
104750	346	3'-UTR	160.0%	---
104751	347	3'-UTR	60.1%	39.9%
104752	348	3'-UTR	72.4%	27.6%
104753	349	3'-UTR	70.0%	30.0%
104754	350	3'-UTR	115.6%	---
104755	351	3'-UTR	71.7%	28.3%
104756	352	3'-UTR	91.5%	8.5%
104757	353	3'-UTR	85.6%	14.4%
104758	354	3'-UTR	97.6%	2.4%
104759	355	3'-UTR	68.6%	31.4%
104760	356	3'-UTR	182.4%	---
104761	357	3'-UTR	110.9%	---
104762	358	3'-UTR	161.4%	---
104763	359	3'-UTR	102.0%	---
104764	360	3'-UTR	113.5%	---

104765	361	3'-UTR	154.8%	---
104766	362	3'-UTR	126.4%	---
104767	363	3'-UTR	116.1%	---
104768	364	3'-UTR	177.7%	---
104769	365	3'-UTR	89.8%	10.2%
104770	366	3'-UTR	94.3%	5.7%
104771	367	3'-UTR	191.2%	---
104772	368	3'-UTR	80.3%	19.7%
104773	369	3'-UTR	133.9%	---
104774	34	3'-UTR	94.8%	5.2%
104775	370	3'-UTR	80.6%	19.4%
104776	371	3'-UTR	90.1%	9.9%
104777	372	3'-UTR	84.7%	15.3%
104778	373	3'-UTR	121.3%	---
104779	374	3'-UTR	97.8%	2.2%
104780	375	3'-UTR	67.6%	32.4%
104781	376	3'-UTR	141.5%	---
104782	377	3'-UTR	96.5%	3.5%
104783	378	3'-UTR	153.2%	---
104784	379	3'-UTR	85.4%	14.6%
104785	380	3'-UTR	163.9%	---
104786	381	3'-UTR	82.9%	17.1%
104787	382	3'-UTR	89.7%	10.3%
104788	383	3'-UTR	103.9%	---
104789	384	3'-UTR	75.8%	24.2%

104790	385	3'-UTR	106.3%	---
104791	386	3'-UTR	165.3%	---
104792	387	3'-UTR	71.8%	28.2%
104793	388	3'-UTR	101.9%	---
104794	389	3'-UTR	70.7%	29.3%
104795	390	3'-UTR	68.8%	31.2%
104796	391	3'-UTR	93.4%	6.6%
104797	37	3'-UTR	131.7%	---
104798	392	3'-UTR	89.4%	10.6%
104799	393	3'-UTR	89.6%	10.4%
104800	394	3'-UTR	89.0%	11.0%
104801	395	3'-UTR	196.8%	---
104802	396	3'-UTR	189.3%	---
104803	397	3'-UTR	119.7%	---
104804	398	3'-UTR	102.4%	---
104805	399	3'-UTR	90.6%	9.4%
104806	400	3'-UTR	89.1%	10.9%
104807	401	3'-UTR	152.6%	---
104808	402	3'-UTR	96.8%	3.2%
104809	403	3'-UTR	178.8%	---
104810	404	3'-UTR	94.9%	5.1%
104811	405	3'-UTR	234.4%	---
104812	406	3'-UTR	114.3%	---
104813	407	3'-UTR	153.7%	---
104814	408	3'-UTR	86.3%	13.7%

104815	409	3'-UTR	153.9%	---
104816	410	3'-UTR	79.9%	20.1%
104817	411	3'-UTR	196.5%	---
104818	412	3'-UTR	94.3%	5.7%
104819	413	3'-UTR	143.3%	---
104820	414	3'-UTR	123.8%	---
104821	415	3'-UTR	129.2%	---
104822	416	5'-UTR	76.6%	23.4%
104823	417	5'-UTR	63.9%	36.1%
104824	418	5'-UTR	22.0%	78.0%
104825	419	5'-UTR	109.4%	---
104826	420	5'-UTR	45.2%	54.8%
104827	421	5'-UTR	68.9%	31.1%
104828	422	5'-UTR	70.9%	29.1%
104829	423	AUG	46.6%	53.4%
104830	424	Coding	55.0%	45.0%
104831	425	Coding	49.5%	50.5%
104832	426	Coding	106.0%	---
104833	427	Coding	23.7%	76.3%
104834	428	Coding	91.8%	8.2%
104835	429	Coding	72.3%	27.7%
104836	430	Coding	63.4%	36.6%
104837	431	Coding	31.0%	69.0%
104838	432	Coding	18.0%	82.0%
104839	433	Coding	67.9%	32.1%

104840	434	Coding	93.8%	6.2%
104841	435	Coding	43.0%	57.0%
104842	436	Coding	73.2%	26.8%
104843	437	Coding	48.1%	51.9%
104844	438	Coding	39.2%	60.8%
104845	439	Coding	37.6%	62.4%
104846	440	Coding	81.7%	18.3%
104847	441	Coding	50.8%	49.2%
104848	442	Coding	56.7%	43.3%
104849	443	Coding	51.8%	48.2%
104850	444	Coding	91.8%	8.2%
104851	445	Coding	93.9%	6.1%
104852	446	Coding	100.9%	---
104853	447	Coding	67.7%	32.3%
104854	23	Coding	11.0%	89.0%
104855	448	Coding	62.5%	37.5%
104856	449	Coding	67.8%	32.2%
104857	92	Coding	28.1%	71.9%
104858	450	Coding	76.2%	23.8%
104859	451	Coding	52.3%	47.7%
104860	452	Coding	93.6%	6.4%
104861	89	Coding	79.3%	20.7%
104862	453	Coding	63.1%	36.9%
104863	454	Stop	64.5%	35.5%
104864	455	Stop	43.2%	56.8%

104865	456	3'-UTR	83.1%	16.9%
104866	457	3'-UTR	49.4%	50.6%
104867	458	3'-UTR	49.5%	50.5%
104868	459	3'-UTR	89.6%	10.4%
104869	460	3'-UTR	21.4%	78.6%
104870	461	3'-UTR	118.0%	---
104871	462	3'-UTR	55.8%	44.2%
104872	463	3'-UTR	49.0%	51.0%
104873	464	3'-UTR	92.6%	7.4%
104874	465	3'-UTR	33.4%	66.6%
104875	466	3'-UTR	36.2%	63.8%
104876	467	3'-UTR	73.4%	26.6%
104877	468	3'-UTR	40.9%	59.1%
104878	469	3'-UTR	78.7%	21.3%
104879	470	3'-UTR	75.4%	24.6%
104880	471	3'-UTR	50.2%	49.8%
104881	472	3'-UTR	47.0%	53.0%
104882	473	3'-UTR	82.7%	17.3%
104883	474	3'-UTR	46.4%	53.6%
104884	475	3'-UTR	46.1%	53.9%
104885	476	3'-UTR	156.9%	---
104886	477	3'-UTR	102.4%	---
104887	478	3'-UTR	59.1%	40.9%
104888	479	3'-UTR	64.7%	35.3%
104889	480	3'-UTR	83.7%	16.3%

104890	481	3'-UTR	52.9%	47.1%
104891	482	3'-UTR	87.9%	12.1%
104892	483	3'-UTR	39.8%	60.2%
104893	484	3'-UTR	71.1%	28.9%
104894	485	3'-UTR	34.0%	66.0%
104895	486	3'-UTR	129.8%	---
104896	487	3'-UTR	57.6%	42.4%
104897	488	3'-UTR	49.6%	50.4%
104898	489	3'-UTR	71.7%	28.3%
104899	490	3'-UTR	101.5%	---
104900	491	3'-UTR	142.1%	---
104901	492	3'-UTR	55.9%	44.1%
104902	493	3'-UTR	85.3%	14.7%
104903	494	3'-UTR	46.0%	54.0%
104904	495	3'-UTR	59.9%	40.1%
104905	496	3'-UTR	47.2%	52.8%
104906	497	3'-UTR	56.3%	43.7%

Oligonucleotides 104662 (SEQ ID NO: 264), 104669 (SEQ ID NO: 271), 104670 (SEQ ID NO: 272), 104688 (SEQ ID NO: 290), 104695 (SEQ ID NO: 297), 104697 (SEQ ID NO: 299), 104713 (SEQ ID NO: 315), 104738 (SEQ ID NO: 334), 104824 (SEQ ID NO: 418),
5 104826 (SEQ ID NO: 420), 104829 (SEQ ID NO: 423), 104831 (SEQ ID NO: 425), 104833 (SEQ ID NO: 427), 104837 (SEQ ID NO: 431), 104838 (SEQ ID NO: 432), 104841 (SEQ ID NO: 435), 104843 (SEQ ID NO: 437), 104844 (SEQ ID NO: 438), 104845 (SEQ ID NO: 439),
10 104847 (SEQ ID NO: 441), 104854 (SEQ ID NO: 23), 104857 (SEQ ID NO: 92), 104864 (SEQ ID NO: 455), 104866 (SEQ ID NO: 457), 104867 (SEQ ID NO: 458), 104869 (SEQ ID NO: 460), 104872 (SEQ

ID NO: 463), 104874 (SEQ ID NO: 465), 104875 (SEQ ID NO: 466), 104877 (SEQ ID NO: 468), 104880 (SEQ ID NO: 471), 104881 (SEQ ID NO: 472), 104883 (SEQ ID NO: 474), 104884 (SEQ ID NO: 475), 104892 (SEQ ID NO: 483), 104894 (SEQ ID NO: 485), 104897 (SEQ ID NO: 488), 104903 (SEQ ID NO: 494) and 104905 (SEQ ID NO: 496) gave approximately 50% or greater reduction in TNF- α mRNA expression in this assay. Oligonucleotides 104713 (SEQ ID NO: 315), 104824 (SEQ ID NO: 418), 104833 (SEQ ID NO: 427), 104837 (SEQ ID NO: 431), 104838 (SEQ ID NO: 432), 104854 (SEQ ID NO: 23), 104857 (SEQ ID NO: 92), and 104869 (SEQ ID NO: 460) gave approximately 70% or greater reduction in TNF- α mRNA expression in this assay.

EXAMPLE 25

15 Dose response of chimeric (deoxy gapped) antisense phosphorothioate oligodeoxynucleotide effects on TNF- α mRNA and protein levels

Several oligonucleotides from the initial screen were chosen for dose response assays. NeoHk cells were grown, treated and processed as described in Example 3. LIPOFECTIN7 was added at a ratio of 3 μ g/ml per 100 nM of oligonucleotide. The control included LIPOFECTIN7 at a concentration of 9 μ g/ml.

The human promonocytic leukaemia cell line, THP-1 (American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 growth media supplemented with 10% fetal calf serum (FCS; Life Technologies, Rockville, MD). A total of 8×10^5 cells were employed for each treatment by combining 50 μ l of cell suspension in OPTIMEMTM, 1% FBS with oligonucleotide at the indicated concentrations to reach a final volume of 100 μ l with OPTIMEMTM, 1% FBS. Cells were then transferred to a 1 mm electroporation cuvette and electroporated using an Electrocell Manipulator 600 instrument

(Biotechnologies and Experimental Research, Inc.) employing 90 V, 1000 μ F, at 13 Ω . Electroporated cells were then transferred to 24 well plates. 400 μ l of RPMI 1640, 10% FCS was added to the cells and the cells were allowed to recover for 6 hrs. Cells were then induced with LPS at a final concentration of 100 ng/ml for 2 hours. RNA was isolated and processed as described in Example 3. Results with NeoHK cells are shown in Table 38 for mRNA, and Table 39 for protein. Results with THP-1 cells are shown in Table 40.

Most of the oligonucleotides tested showed dose response effects with a maximum inhibition of mRNA greater than 70% and a maximum inhibition of protein greater than 85%.

TABLE 38

Dose Response of NeoHK Cells to TNF- α
Chimeric (deoxy gapped) Antisense Oligonucleotides

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
induced	---	---	---	100%	---
16798	128	coding	30 nM	87%	13%
"	"	"	100 nM	129%	---
"	"	"	300 nM	156%	---
21823	69	intron 1	30 nM	82%	18%
"	"	"	100 nM	90%	10%
"	"	"	300 nM	59%	41%
28088	68	intron 1	30 nM	68%	32%
"	"	"	100 nM	43%	57%
"	"	"	300 nM	42%	58%
28089	69	intron 1	30 nM	59%	41%

"	"	"	100 nM	44%	56%
"	"	"	300 nM	38%	62%
104697	299	coding	30 nM	60%	40%
"	"	"	100 nM	45%	55%
"	"	"	300 nM	27%	73%
104777	372	3'-UTR	30 nM	66%	34%
"	"	"	100 nM	55%	45%
"	"	"	300 nM	43%	57%

TABLE 39

Dose Response of NeoHK Cells to TNF- α
Chimeric (deoxy gapped) Antisense Oligonucleotides

5

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% Protein Expression	% Protein Inhibition
induced	---	---	---	100.0%	---
16798	128	coding	30 nM	115.0%	---
"	"	"	100 nM	136.0%	---
"	"	"	300 nM	183.0%	---
28089	69	intron 1	30 nM	87.3%	12.7%
"	"	"	100 nM	47.4%	52.6%
"	"	"	300 nM	22.8%	77.2%
104681	283	coding	30 nM	91.3%	8.7%
"	"	"	100 nM	62.0%	38.0%
"	"	"	300 nM	28.5%	71.5%
104697	299	coding	30 nM	87.1%	12.9%
"	"	"	100 nM	59.6%	40.4%

"	"	"	300 nM	29.1%	70.9%
104838	432	coding	30 nM	91.9%	8.1%
"	"	"	100 nM	56.9%	43.1%
"	"	"	300 nM	14.8%	85.2%
104854	23	coding	30 nM	64.4%	35.6%
"	"	"	100 nM	42.3%	57.7%
"	"	"	300 nM	96.1%	3.9%
104869	460	3'-UTR	30 nM	88.9%	11.1%
"	"	"	100 nM	56.8%	43.2%
"	"	"	300 nM	42.3%	57.7%

TABLE 40

5

**Dose Response of LPS-Induced THP-1 Cells to Chimeric
(deoxy gapped) TNF- α Antisense Phosphorothioate
Oligodeoxynucleotides (ASOs)**

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
induced	---	---	---	100%	---
16798	128	coding	1 μ M	102%	--
"	"	"	3 μ M	87%	13%
"	"	"	10 μ M	113%	---
"	"	"	30 μ M	134%	---
28089	69	intron 1	1 μ M	39%	61%
"	"	"	3 μ M	79%	21%
"	"	"	10 μ M	91%	9%
"	"	"	30 μ M	63%	37%

104697	299	coding	1 μ M	99%	1%
"	"	"	3 μ M	96%	4%
"	"	"	10 μ M	92%	8%
"	"	"	30 μ M	52%	48%
104838	432	coding	1 μ M	31%	69%
"	"	"	3 μ M	20%	80%
"	"	"	10 μ M	15%	85%
"	"	"	30 μ M	7%	93%
104854	23	coding	1 μ M	110%	---
"	"	"	3 μ M	90%	10%
"	"	"	10 μ M	95%	5%
"	"	"	30 μ M	61%	39%

EXAMPLE 26**Further Optimization of Human TNF- α Antisense Oligonucleotide Chemistry**

5 Additional analogs of TNF- α oligonucleotides were designed and synthesized to find an optimum gap size. The sequences and chemistries are shown in Table 36.

Dose response experiments are performed as described in Example 3.

TABLE 41

**Nucleotide Sequences of TNF- α Chimeric Backbone (deoxy gapped)
Oligonucleotides**

5

ISIS NO.	NUCLEOTIDE SEQUENCE ¹ (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES ²	GENE TARGET REGION
110554	GCTGATTAGAGAGAGGTCCC	432	104838 analog	
110555	GCTGATTAGAGAGAGGTCCC	"	"	
110556	GCTGATTAGAGAGAGGTCCC	"	"	
110557	GCTGATTAGAGAGAGGTCCC	"	"	
110583	GCTGATTAGAGAGAGGTCCC	"	"	
110558	CTGATTAGAGAGAGGTCCC	498	1596-1614	coding
110559	CTGATTAGAGAGAGGTCCC	"	"	"
110560	CTGATTAGAGAGAGGTCCC	"	"	"
110561	CTGATTAGAGAGAGGTCCC	"	"	"
110562	CTGATTAGAGAGAGGTCCC	"	"	"
110563	CTGATTAGAGAGAGGTCCC	"	"	"
110564	CTGATTAGAGAGAGGTCCC	"	"	"
110565	CTGATTAGAGAGAGGTCCC	"	"	"
110566	CTGATTAGAGAGAGGTCCC	"	"	"
110567	CTGATTAGAGAGAGGTCCC	"	"	"
110584	CTGATTAGAGAGAGGTCCC	"	"	"
108371	CTGATTAGAGAGAGGTCC	499	1597-1614	coding
110568	CTGATTAGAGAGAGGTCC	"	"	"

110569	CTGATTAGAGAGAGGTCC	"	"	"
110570	CTGATTAGAGAGAGGTCC	"	"	"
110585	CTGATTAGAGAGAGGTCC	"	"	"
110571	CTGGTTATCTCTCAGCTCCA	299	104697 analog	
110572	CTGGTTATCTCTCAGCTCCA	"	"	
110573	CTGGTTATCTCTCAGCTCCA	"	"	
110586	CTGGTTATCTCTCAGCTCCA	"	"	
110574	GATCACTCCAAAGTGCAGCA	283	104681 analog	
110575	GATCACTCCAAAGTGCAGCA	"	"	
110576	GATCACTCCAAAGTGCAGCA	"	"	
110587	GATCACTCCAAAGTGCAGCA	"	"	
110577	AGCTTGGGTTCCGACCCTAA	460	104689 analog	
110578	AGCTTGGGTTCCGACCCTAA	"	"	
110579	AGCTTGGGTTCCGACCCTAA	"	"	
110588	AGCTTGGGTTCCGACCCTAA	"	"	
110580	AGGTTGACCTTGGTCTGGTA	315	104713 analog	
110581	AGGTTGACCTTGGTCTGGTA	"	"	
110582	AGGTTGACCTTGGTCTGGTA	"	"	
110589	AGGTTGACCTTGGTCTGGTA	"	"	
110637	GTGTGCCAGACACCCTATCT	69	21823 analog	
110651	GTGTGCCAGACACCCTATCT	"	"	
110665	GTGTGCCAGACACCCTATCT	"	"	
110679	GTGTGCCAGACACCCTATCT	"	"	
110693	GTGTGCCAGACACCCTATCT	"	"	

110707	GTGTGCCAGACACCCTATCT	"	"	
110590	TGAGTGTCTTCTGTGTGCCA	500	1411-1430	intron 1
110597	TGAGTGTCTTCTGTGTGCCA	"	"	"
110604	TGAGTGTCTTCTGTGTGCCA	"	"	"
110611	TGAGTGTCTTCTGTGTGCCA	"	"	"
110618	TGAGTGTCTTCTGTGTGCCA	"	"	"
110625	TGAGTGTCTTCTGTGTGCCA	"	"	"
110591	GAGTGTCTTCTGTGTGCCAG	501	1410-1429	intron 1
110598	GAGTGTCTTCTGTGTGCCAG	"	"	"
110605	GAGTGTCTTCTGTGTGCCAG	"	"	"
110612	GAGTGTCTTCTGTGTGCCAG	"	"	"
110619	GAGTGTCTTCTGTGTGCCAG	"	"	"
110626	GAGTGTCTTCTGTGTGCCAG	"	"	"
110592	AGTGTCTTCTGTGTGCCAGA	144	100181 analog	
110599	AGTGTCTTCTGTGTGCCAGA	"	"	
110606	AGTGTCTTCTGTGTGCCAGA	"	"	
110613	AGTGTCTTCTGTGTGCCAGA	"	"	
110620	AGTGTCTTCTGTGTGCCAGA	"	"	
110627	AGTGTCTTCTGTGTGCCAGA	"	"	
110593	GTGTCTTCTGTGTGCCAGAC	145	100182 analog	
110600	GTGTCTTCTGTGTGCCAGAC	"	"	
110607	GTGTCTTCTGTGTGCCAGAC	"	"	
110614	GTGTCTTCTGTGTGCCAGAC	"	"	
110621	GTGTCTTCTGTGTGCCAGAC	"	"	

110628	GTGTCTTCTGTGTGCCAGAC	"	"	
110594	TGTCTTCTGTGTGCCAGACA	146	100183 analog	
110601	TGTCTTCTGTGTGCCAGACA	"	"	
110608	TGTCTTCTGTGTGCCAGACA	"	"	
110615	TGTCTTCTGTGTGCCAGACA	"	"	
110622	TGTCTTCTGTGTGCCAGACA	"	"	
110629	TGTCTTCTGTGTGCCAGACA	"	"	
110595	GTCTTCTGTGTGCCAGACAC	147	100184 analog	
110602	GTCTTCTGTGTGCCAGACAC	"	"	
110609	GTCTTCTGTGTGCCAGACAC	"	"	
110616	GTCTTCTGTGTGCCAGACAC	"	"	
110623	GTCTTCTGTGTGCCAGACAC	"	"	
110630	GTCTTCTGTGTGCCAGACAC	"	"	
110596	TCTTCTGTGTGCCAGACACC	148	100185 analog	
110603	TCTTCTGTGTGCCAGACACC	"	"	
110610	TCTTCTGTGTGCCAGACACC	"	"	
110617	TCTTCTGTGTGCCAGACACC	"	"	
110624	TCTTCTGTGTGCCAGACACC	"	"	
110631	TCTTCTGTGTGCCAGACACC	"	"	
110632	CTTCTGTGTGCCAGACACCC	149	100186 analog	
110646	CTTCTGTGTGCCAGACACCC	"	"	
110660	CTTCTGTGTGCCAGACACCC	"	"	
110674	CTTCTGTGTGCCAGACACCC	"	"	
110688	CTTCTGTGTGCCAGACACCC	"	"	

110702	CTTCTGTGTGCCAGACACCC	"	"	
110633	TTCTGTGTGCCAGACACCCT	150	100187 analog	
110647	TTCTGTGTGCCAGACACCCT	"	"	
110661	TTCTGTGTGCCAGACACCCT	"	"	
110675	TTCTGTGTGCCAGACACCCT	"	"	
110689	TTCTGTGTGCCAGACACCCT	"	"	
110703	TTCTGTGTGCCAGACACCCT	"	"	
110634	TCTGTGTGCCAGACACCCTA	151	100188 analog	
110648	TCTGTGTGCCAGACACCCTA	"	"	
110662	TCTGTGTGCCAGACACCCTA	"	"	
110676	TCTGTGTGCCAGACACCCTA	"	"	
110690	TCTGTGTGCCAGACACCCTA	"	"	
110704	TCTGTGTGCCAGACACCCTA	"	"	
110635	CTGTGTGCCAGACACCCTAT	152	100189 analog	
110649	CTGTGTGCCAGACACCCTAT	"	"	
110663	CTGTGTGCCAGACACCCTAT	"	"	
110677	CTGTGTGCCAGACACCCTAT	"	"	
110691	CTGTGTGCCAGACACCCTAT	"	"	
110705	CTGTGTGCCAGACACCCTAT	"	"	
110636	TGTGTGCCAGACACCCTATC	153	100190 analog	
110650	TGTGTGCCAGACACCCTATC	"	"	
110664	TGTGTGCCAGACACCCTATC	"	"	
110678	TGTGTGCCAGACACCCTATC	"	"	
110692	TGTGTGCCAGACACCCTATC	"	"	

110706	TGTGTGCCAGACACCCTATC	"	"	
110638	TGTGCCAGACACCCTATCTT	154	100191 analog	
110652	TGTGCCAGACACCCTATCTT	"	"	
110666	TGTGCCAGACACCCTATCTT	"	"	
110680	TGTGCCAGACACCCTATCTT	"	"	
110694	TGTGCCAGACACCCTATCTT	"	"	
110708	TGTGCCAGACACCCTATCTT	"	"	
110639	GTGCCAGACACCCTATCTTC	155	100192 analog	
110653	GTGCCAGACACCCTATCTTC	"	"	
110667	GTGCCAGACACCCTATCTTC	"	"	
110681	GTGCCAGACACCCTATCTTC	"	"	
110695	GTGCCAGACACCCTATCTTC	"	"	
110709	GTGCCAGACACCCTATCTTC	"	"	
110640	TGCCAGACACCCTATCTTCT	156	100193 analog	
110654	TGCCAGACACCCTATCTTCT	"	"	
110668	TGCCAGACACCCTATCTTCT	"	"	
110682	TGCCAGACACCCTATCTTCT	"	"	
110696	TGCCAGACACCCTATCTTCT	"	"	
110710	TGCCAGACACCCTATCTTCT	"	"	
110641	GCCAGACACCCTATCTTCTT	157	100194 analog	
110655	GCCAGACACCCTATCTTCTT	"	"	
110669	GCCAGACACCCTATCTTCTT	"	"	
110683	GCCAGACACCCTATCTTCTT	"	"	
110697	GCCAGACACCCTATCTTCTT	"	"	

110711	GCCAGACACCCTATCTTCTT	"	"	
110642	CCAGACACCCTATCTTCTTC	158	100195 analog	
110656	CCAGACACCCTATCTTCTTC	"	"	
110670	CCAGACACCCTATCTTCTTC	"	"	
110684	CCAGACACCCTATCTTCTTC	"	"	
110698	CCAGACACCCTATCTTCTTC	"	"	
110712	CCAGACACCCTATCTTCTTC	"	"	
110643	CAGACACCCTATCTTCTTCT	159	100196 analog	
110657	CAGACACCCTATCTTCTTCT	"	"	
110671	CAGACACCCTATCTTCTTCT	"	"	
110685	CAGACACCCTATCTTCTTCT	"	"	
110699	CAGACACCCTATCTTCTTCT	"	"	
110713	CAGACACCCTATCTTCTTCT		"	
110644	AGACACCCTATCTTCTTCTC	160	100197 analog	
110658	AGACACCCTATCTTCTTCTC	"	"	
110672	AGACACCCTATCTTCTTCTC	"	"	
110686	AGACACCCTATCTTCTTCTC	"	"	
110700	AGACACCCTATCTTCTTCTC	"	"	
110714	AGACACCCTATCTTCTTCTC	"	"	
110645	GACACCCTATCTTCTTCTCT	161	100198 analog	
110659	GACACCCTATCTTCTTCTCT	"	"	
110673	GACACCCTATCTTCTTCTCT	"	"	
110687	GACACCCTATCTTCTTCTCT	"	"	

110701	GACACCCTATCTTCTTCTCT	"	"	
110715	GACACCCTATCTTCTTCTCT	"	"	

¹ Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines and 2'-deoxycytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

²Co-ordinates from Genbank Accession No. X02910, locus name "HSTNFA", SEQ ID NO. 1.

10

EXAMPLE 26**Effect of TNF- α antisense oligonucleotides in TNF- α transgenic mouse models**

The effect of TNF- α antisense oligonucleotides is studied in transgenic mouse models of human diseases. Such experiments can be performed through contract laboratories (e.g., The Laboratory of Molecular Genetics at The Hellenic Pasteur Institute, Athens, Greece) where such transgenic mouse models are available. Such models are available for testing human oligonucleotides in arthritis (Keffer, J., et al., *EMBO J.*, **1991**, *10*, 4025-4031) and multiple sclerosis (Akassoglou et al., *J. Immunol.*, **1997**, *158*, 438-445) models. A model for inflammatory bowel disease is available for testing mouse oligonucleotides (Kontoyiannis et al., *Immunity*, **1999**, *10*, 387-398).

Briefly, litters of the appropriate transgenic mouse strain are collected and weighed individually. Twice weekly from birth, oligonucleotide in saline is administered intraperitoneally or intravenously. Injections continue for 7 weeks. Each week the animals are scored for manifestations of the appropriate disease. After the final treatment, the mice are sacrificed and histopathology is performed for indicators of disease as indicated in the references cited for each model.

35

Example 27**Design and screening of duplexed antisense compounds targeting TNF- α**

In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements can be designed to target TNF- α . The nucleobase sequence of the antisense strand of the duplex comprises at least a portion of an oligonucleotide to TNF- α as described herein. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini. For example, a duplex comprising an antisense strand having the sequence CGAGAGGCGGACGGGACCG and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

cgagaggcggacgggaccgTT	Antisense Strand
TTgctctccgctgccctggc	Complement

RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacon Research Inc., (Lafayette, CO). Once synthesized, the complementary strands are annealed. The single strands are aliquoted and diluted to a concentration of 50 uM. Once diluted, 30 uL of each strand is combined with 15uL of a 5X solution of annealing buffer. The final concentration of said buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2mM magnesium acetate. The final volume is 75 uL. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The tube is allowed to sit for 1 hour at 37°C at which time the dsRNA duplexes are used in experimentation. The final concentration

of the dsRNA duplex is 20 uM. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate TNF- α expression according to the protocols described herein.

Example 28

Design of phenotypic assays and in vivo studies for the use of TNF- α inhibitors

10 *Phenotypic assays*

Once TNF- α inhibitors have been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition.

Phenotypic assays, kits and reagents for their use are well known to those skilled in the art and are herein used to investigate the role and/or association of TNF- α in health and disease. Representative phenotypic assays, which can be purchased from any one of several commercial vendors, include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based assays including enzymatic assays (Panvera, LLC, Madison, WI; BD Biosciences, Franklin Lakes, NJ; Oncogene Research Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for

obesity studies) are treated with TNF- α inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated
5 and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of
10 cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest. Analysis of the genotype of the cell
15 (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the efficacy or potency of the TNF- α inhibitors. Hallmark genes, or those genes suspected to be associated with a specific disease state, condition, or phenotype, are measured in both
20 treated and untreated cells.